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ANNALS OF BOTANY

NEW SERIES. VOLUME I

ANNALS OF BOTANY

EDITED BY

V. H. BLACKMAN, Sc.D., F.R.S.
PROFESSOR OF PLANT PHYSIOLOGY, IMPERIAL COLLEGE
OF SCIENCE AND TECHNOLOGY, LONDON

ASSISTED BY

A. J. EAMES, Ph.D.
PROFESSOR OF BOTANY, CORNELL UNIVERSITY
ITHACA, N.Y., U.S.A.

Sir JOHN FARMER, M.A., LL.D., D.Sc., F.R.S.
EMERITUS PROFESSOR OF BOTANY, IMPERIAL COLLEGE OF SCIENCE
AND TECHNOLOGY, LONDON

F. W. OLIVER, M.A., D.Sc., F.R.S.
EMERITUS PROFESSOR OF BOTANY
UNIVERSITY COLLEGE, LONDON

AND OTHER BOTANISTS

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Foreword to the New Series Vol. I (51st Volume)

THE completion of its first half-century of publication may be said to mark a noteworthy epoch in the life of a scientific (and perhaps of any other) periodical, and as one who was more or less closely in contact with the chief promotors of *The Annals of Botany* during that somewhat remote period of its inception, it has been suggested that I should write this foreword on the occasion of its jubilee and the beginning of a New Series.

Although only an undergraduate at Oxford at that time, I enjoyed the intimate friendship of Professor Isaac Bayley Balfour, to whose vigour and vitalizing energy the inception of the enterprise was so largely due.

A strong committee was formed, of whom Balfour, Vines, and Marshall Ward were prominent members. On January 20, 1887, a letter signed by the two first-named botanists was dispatched to the secretary of the Delegates of the Clarendon Press, Oxford, with the view of opening negotiations and exploring the conditions on which it would be possible to inaugurate the enterprise. In this letter the title proposed for the new venture was 'The Journal of Botanical Science'. The Clarendon Press was sympathetic, but not unnaturally stipulated for a guarantee against loss should the venture prove unsuccessful.

This condition was agreed to, and a sum regarded as adequate was easily forthcoming.

At some stage in the course of this incubation period the name was changed to that of 'The Annals of Botany', but on whose suggestion this excellent alteration was made I have been unable to discover.

The first number of *The Annals* appeared in August, 1887, and the three editors responsible for its production were Balfour (Oxford), Vines (Cambridge), and Farlow (Harvard, U.S.A.) and this happy association with the United States has been perpetuated throughout the half century of its existence.

The financial success of *The Annals* was naturally a matter of some anxiety to its original promotors, and it is very satisfactory to know that by 1895 it had become clear that the new journal was to pay its way. Three years later the Clarendon Press returned the whole of the guarantee fund which had been deposited with them.

The only material change in the character of *The Annals* since its first appearance has consisted in the disappearance of the 'Record of Current Literature' from the fifth and all subsequent volumes, and its replacement by additional original papers. Probably most, if not all, subscribers would agree that the exchange has been fully justified.

J. B. FARMER

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The Effect on the Behaviour of Stomata of Alternating Periods of Light and Darkness of Short Duration

BY

F. G. GREGORY

AND

H. L. PEARSE

(From the Research Institute of Plant Physiology, Imperial College of Science and Technology, London, S.W. 7)

With three Figures in the Text

INTRODUCTION

SO far as the authors are aware, with the exception of a few incidental observations by Paetz (1930), no investigations have been made on the effect of alternating periods of light and darkness of short duration on the behaviour of stomata. Garner and Allard (1931), in the course of their investigation on photoperiodism, grew plants of various species in alternating periods of light and darkness of very different durations, varying from twelve hours to five seconds. Very great differences in vegetative growth were obtained with varying durations of alternation, a marked minimum in vegetative growth being apparent in the neighbourhood of alternating periods of light and darkness of one minute. Either increase or decrease in duration of the alternating periods from this minimal point resulted in an increase in growth. No explanation of this profound effect of short alternating periods was offered by these observers. It occurred to one of the present authors that possibly stomatal closure was concerned in this effect, and preliminary observations with alternations of light and darkness of one minute duration confirmed this conjecture. An experiment performed by Portsmouth (1937) with *Cucumis sativus* grown under controlled conditions of temperature and humidity with artificial light has confirmed the observations of Garner and Allard. Cucumber plants grown with twelve hours alternations of light and darkness showed considerably greater vegetative development, both in dry weight and leaf area, than did plants receiving alternate periods of one minute duration. An automatic record of stomatal movement showed a greater aperture in the long light periods than in the short.

It seemed of interest to investigate further this effect of short alternating light and dark periods, more particularly as it seemed to offer some possibility of analysing further the relation of the opening and closing mechanisms of the stomata.

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METHOD

The plant used for all these experiments was *Pelargonium zonale* (var. Paul Crampel). The plants which had been raised in the greenhouse from cuttings were given no special treatment before use. They were taken direct from the greenhouse and placed in a light-proof box, and illuminated by a 100-watt lamp, which was cooled with a running water screen; the temperature of the box was maintained nearly constant at 25° C. by means of electric heaters consisting of resistance mats distributed uniformly over the floor of the chamber. The pot containing the plant was supported some distance from the floor on a double layer of asbestos sheet and thus was never unduly heated. Temperature control was effected with a Hearson capsule.

The behaviour of the stomata during the course of an experiment was kept under continual observation by the automatic recording modification of the Resistance Porometer previously described by Gregory and Pearse (1934). In this method the air is drawn through the leaf into a porometer cup by means of a constant pressure aspirator. Between the porometer cup and the aspirator a capillary tube of convenient flow resistance is inserted and a water manometer records the varying pressure in the system at a point between the porometer cup and the standard resistance. If R_2 represents the flow resistance of the stomata, R_1 the flow resistance of the standard capillary, P_2 the pressure difference in the manometer, and P_1 the constant head of pressure in the aspirator; then the flow resistance R_2 of the stomata is determined by the relation:

$$R_2 = \frac{P_2 R_1}{P_1 - P_2}.$$

It has been shown that for maximum sensitivity the value of R_1 should be such that $R_1 = R_2$. Actually the value of R_1 used was approximately equal to the flow resistance of the half-opened stomata. The level of water in the manometer was recorded by passing a parallel beam of heat-rays from a lamp through one limb of the manometer, and in determining the energy transmitted by means of a low-resistance thermopile and a Cambridge recording millivoltmeter. It has been shown that the reading of the millivoltmeter over a wide range of stomatal opening is proportional to the manometer reading.

The porometer cup was attached to the leaf by means of a washer of 30 per cent. gelatine, the requisite pressure necessary to obtain an air-tight joint being effected by clamping the cup gently to a glass plate against which the leaf was pushed.

In carrying out an experiment the following procedure was adopted. After attaching the porometer cup the leaf was allowed to recover in the dark from the shock, and was then given a period of continuous light during which the stomata opened to their maximum aperture. This was followed generally by a period of intermittent illumination of varying duration followed by a period of darkness and a second period of alternating light. The varying light régime was carried through automatically by means of the apparatus shown diagrammatically in Fig. 1.

D represents a metal clockwork drum rotating once in twenty-four hours. On the surface of this are three sliding contacts, A, B, and C, which either make

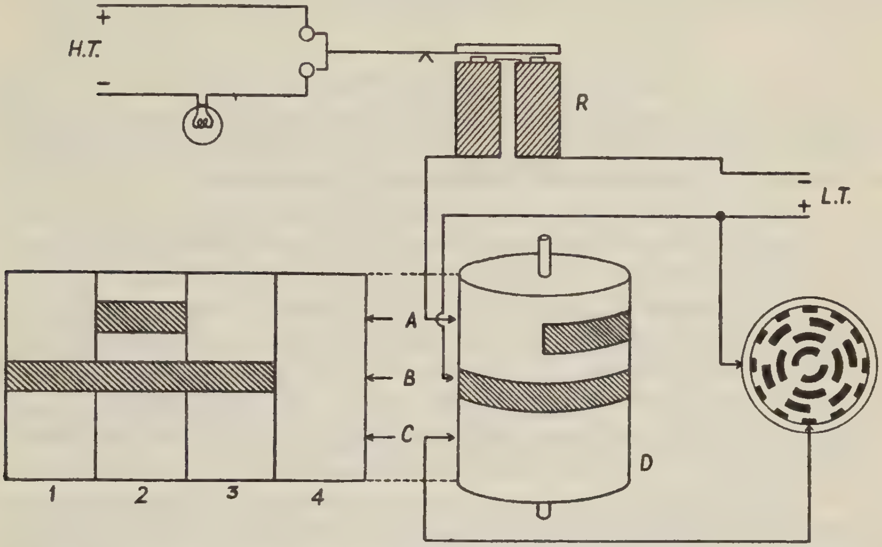


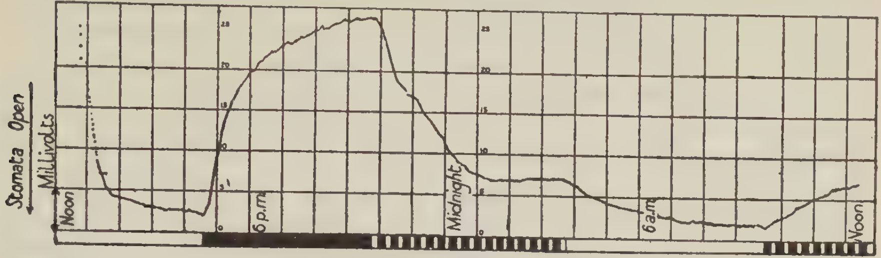
FIG. 1. Diagram of circuit used for obtaining automatically the sequence of lighting conditions during the experiments. For explanation see text.

electrical contact or are insulated from the surface by strips of celluloid. I represents an interrupter revolving once in four minutes. This consists in a large metal disc driven by means of a constant speed electric motor, carrying on its surface two contact wheels, one of which runs on the metal surface and the other runs over a series of insulating strips so that the current is interrupted at regular intervals. A number of such circles of insulating strips with different numbers of segments were mounted on the disc, so that by altering the position of the contact wheel, varying periods of alternation were obtained. In this way alternating periods varying from five seconds to two minutes were obtained. When necessary the speed of rotation of the disc was changed by means of a rheostat controlling the current to the motor. The use of the three contacts on the control drum D can be seen from the diagram. The source of low-tension current was used to activate the relay R which controlled the high-tension circuit, in which the lamp in the plant chamber was included. When the relay R was activated the current in the high-tension circuit was broken and the plant was in darkness. The strips of celluloid on the surface of the drum D are shown in plan in the diagram. The surface of the drum consists of four portions, as numbered. When the contacts (A, B, C) rest on part 4 the interrupter is short-circuited and the relay is activated so that this represents a period of continuous darkness. When parts 1 and 3 are in use the contact B is broken so that the current now passes through the interrupter and supplies intermittent illumination to the plant chamber. When contact is made with part 2 the low-tension circuit is completely broken and the plant then receives continuous light. By starting at a convenient position on the drum, therefore, the variations in illumination required were automatically obtained.

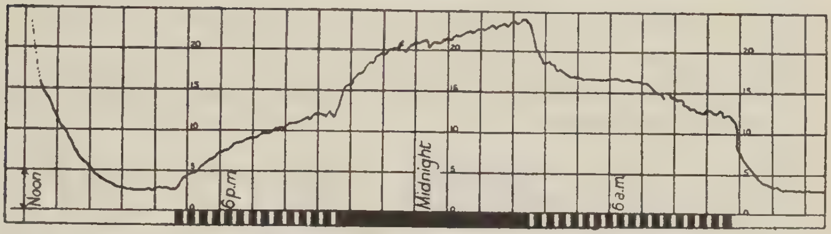
EXPERIMENTAL RESULTS

In Fig. 2 are shown automatic records of four typical experiments. The periods of alternation shown are two minutes, one minute, forty-five seconds, and five seconds respectively. The millivoltmeter recorded the water-level in the manometer at intervals of one minute, the records on the chart being represented by points. For purposes of reproducing the charts these points have been joined together by a continuous line, which, however, faithfully represents the original record. Only in the preliminary opening in two cases are the individual points in the chart shown. The abscissae in the diagram show time-intervals of one hour, and the actual time of day is indicated. The diurnal variation in the minimal stomatal aperture reached, which was previously noted in Pelargonium (Gregory and Pearse, 1934) under conditions of long periods of three-hourly alternations with artificial light, was confirmed in these experiments. It was therefore necessary to begin the experiments at the same hour each day. In the charts the two periods of alternating light are shown, and in general one continuous light and dark period also. The effect of alternating light is thus seen immediately following a continuous light and a continuous dark period. There is considerable variation among the plants of the different experiments, both in the rate and the degree of the opening in continuous light, but by the end of this period the stomata have reached a nearly constant aperture. The variations between plants may to some extent be accounted for by variation in the effective area of the porometer cup, since slight differences in pressure on the leaf lead to differences in spread of the gelatine washer. During the period of alternations immediately after continuous light, in every case, the stomata show a closing movement, the extent of change depending on the period of alternation. On darkening the plants a further rapid closure takes place and the stomata return to their original closed position. During this dark period there are very clear indications of rhythmical fluctuations in stomatal aperture. This is seen clearly in the experiments with one minute and 5 seconds alternations, and in Fig. 3 one of many other records is presented in which plants, kept in continuous light for a long period, showed this phenomenon for more than twelve hours. The period of fluctuation is in general in the neighbourhood of a quarter of an hour for a complete period. Slow rhythmical oscillations in stomatal aperture of approximately twenty minute periods have been recorded in *Vicia Faba* by direct microscopic observation by Stalfelt (1929), although these observations have been called in question by Kerl (1929) and Boresch (1933).

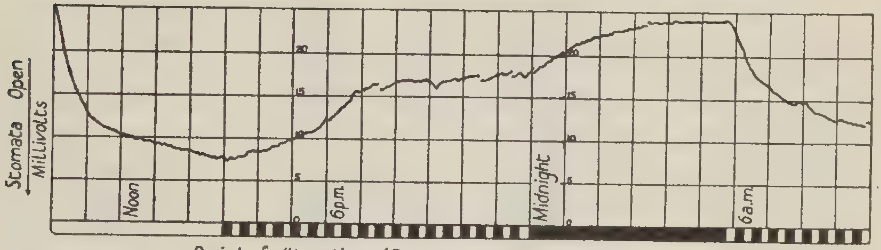
In the alternating period following the dark period opening of the stomata occurs, and it will be noted from the graphs that in general the equilibrium of stomatal aperture reached in the alternating light is the same whether approached from the position of maximal opening or closure. From all the data available the relation of the equilibrium aperture in alternating light of varying duration to the maximal opening has been calculated. The flow



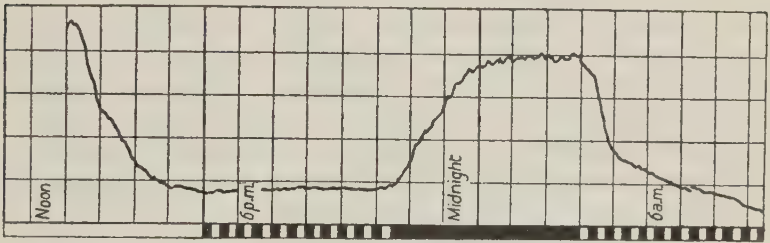
Period of Alternation - 2mins



Period of Alternation - 1 min



Period of Alternation - 45 secs



Period of Alternation - 5 sec

FIG. 2. Automatic record of stomatal movement with different alternating periods of light and darkness as indicated. Two periods of alternating light are shown and also continuous light and darkness.

resistance of the stomata at the end of continuous light and alternating light periods respectively was determined, and the ratio of these two resistances was found. The relevant data are given in the Table. It will be seen that, starting

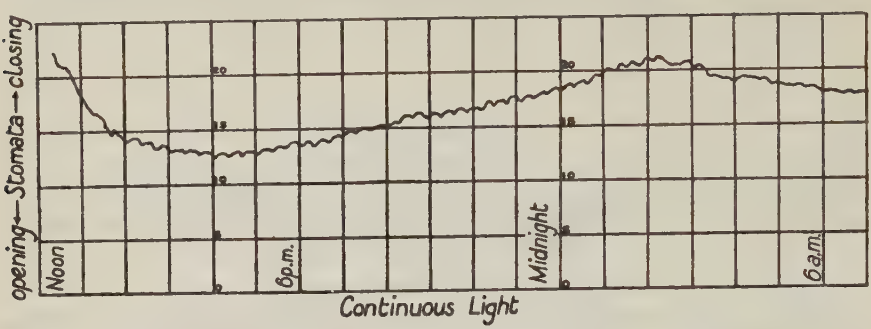


FIG. 3. Automatic record of stomatal movement in continuous light showing autonomous pulsations of the stomata. Note also autonomous closure reaching a minimum aperture soon after midnight.

Period of alternations.	Resistance in continuous light. Capillary units.	Resistance in alternating period. Capillary units.	Ratio of Col. 3 / Col. 2
2 mins.	0·95	3·3	
	1·0	3·60	3·5
1 „	5.95	7·1	
	5·00	20·0	5·7
45 secs.	3·7	14·5	
	0·95	14·0	9·3
20 „	2·7	10·0	
	3·5	11·5	3·5
10 „	4·4	9·9	
	1·4	5·0	3·0
5 „	1·45	1·8	
	2·35	3·2	1·3

from an alternation of five seconds, on increasing the period of intermittent light the relative aperture in alternating light becomes progressively less, reaching minimum at forty-five seconds and then again increasing. The agreement in the replicate experiments is not very satisfactory, so that the position of the minimal aperture with relation to periods of alternation cannot be determined with any great precision. It is interesting to note that the period of intermittent light and darkness giving maximal closure closely approaches the one minute interval noted by Garner and Allard (1931) in their experiments resulting in minimal vegetative growth.

DISCUSSION

It was found extremely difficult to obtain comparable results in these experiments, and it seems likely that many factors other than the direct effect of light play a part in determining the response. In so far as stomatal aperture is related to variation in assimilation the results obtained by Garner and Allard may well have been due to carbohydrate starvation. The sugar analyses performed by Portsmouth (1937) confirm this view. Further, it would appear that the previous history of the plant may be very important, more particularly in relation to the water-supply to which the plant has been subjected. Under continuous light during twenty-four hours the stomata showed no fixed aperture but always tended to close during the night (see Fig. 3), reaching a minimum soon after midnight and then again opening; for this reason, as stated above, the experiments were always carried out at the same period of the day. This spontaneous closure of the stomata under constant conditions indicates some profound internal change not at present understood.

The variation in stomatal aperture in alternating periods of light and dark may be related to the variation in rate of the respective opening and closing reactions. The opening reaction in Pelargonium begins almost instantaneously on illumination, and with a resistance porometer has been noted within one minute; the closing reaction, on the other hand, shows a considerable lag period which varies in extent, but is not less than one minute. If the effects of these reactions are additive it would lead inevitably to the result that, from a position of maximum closure under alternating light, the stomata would open, although at different rates with different periods of light and darkness. The theory, however, fails to account for three observed results, i.e.: (1) stomata at their maximal aperture close under alternating light; (2) the stomata under alternating light reach an equilibrium position which is the same whether approached from maximum closure or opening; (3) the position of this equilibrium is related to the duration of the alternating periods. The theory suggested would result, with any alternation, in a complete opening of the stomata.

The form of the curves of opening and closing throw some light on these observations. During closure, after the preliminary lag period, the stomatal aperture at first decreases rapidly with time, the rate of change progressively becomes slower, the whole resembling a logarithmic decrement curve. The stomatal opening, on the other hand, shows a curve of variation in aperture with time of the sigmoid form. Summations of the gradients, over time-intervals, of these curves at points corresponding with various stomatal apertures, would lead to either preponderance of opening or closing according to the portion of the curves selected and the duration of the interval. The data available are insufficient to predict from these curves the position of the equilibrium point with alternating light, though it is apparent that an equilibrium in each case will be reached at some value of stomatal aperture. The interpretation of the form of these curves presents another problem which need not be discussed at present. It would appear, however, that the reactions concerned in the processes of opening and closing are different in nature.

SUMMARY

The effect of alternating periods of light and darkness on the stomatal movement in the leaf of *Pelargonium zonale* has been studied with periods varying from five seconds to two minutes.

Stomatal aperture was automatically recorded by means of the Recording Resistance Porometer previously described by the authors (Gregory and Pearse, 1934).

Transition from darkness to alternating light leads to partial opening, while passage from full light to alternating leads to partial closure of the stomata.

With each duration of alternating light a fairly definite equilibrium position is reached, and this is the same whether approached from the fully closed or open position of the stomata.

At equilibrium a minimum opening is obtained with alternations of forty-five seconds duration. With both longer and shorter alternations the stomata at equilibrium have a larger aperture.

Evidence for rhythmical variations of the size of the stomata appears in the records. In one record shown (Fig. 3) these are maintained for twelve hours in continuous light. The period of pulsation is approximately fifteen minutes.

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Studies in the Inheritance of Physiological Characters

III. Hybrid Vigour in the Tomato

Part. 1. Manifestations of Hybrid Vigour from Germination to the Onset of Flowering

BY

ERIC ASHBY, D.Sc.

(From the Department of Botany, The University, Bristol)[1]

With eleven Figures in the Text

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INTRODUCTION

THE expressions *hybrid vigour* and *heterosis* are used to describe a variety of phenomena which may appear in the progeny of a cross between inbred parents. The hybrids are often bigger than either parent, they germinate better, they flower earlier, and they yield a bigger harvest of fruit. These phenomena were familiar to horticulturists as early as the eighteenth century, and were described in detail by Kölreuter (1766) and Knight (1799). Occasionally first generation crosses of animals show heterosis: its manifestations in rabbits and guinea-pigs, for example, have been described by Castle (1916).

Until genetics became popular biologists were content to regard heterosis as the result of a 'stimulus'. It was thought that the union of nuclei from dissimilar parents brought about a rejuvenescence of the race (East and Hayes, 1911; Jost, 1907; Shull, 1912). But the particulate theory of inheritance offered a more satisfactory analysis, and Bruce showed in 1910 how heterosis could be attributed to the recombination of characters for vigorous growth. In the same year Keeble and Pellew (1910) showed that the greater size of hybrids between two inbred races of peas was due to a combination

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[1] Most of the data assembled in this paper were collected while the author was on the Staff of the Imperial College of Science and Technology. The experiments were carried out at the John Innes Horticultural Institution.

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in the hybrid of two dominant factors, one from each parent. A factor for thickness of stem in the dwarf, combined with a factor for long stem in the tall, produced a progeny bigger than either. Jones (1918) has shown that this simple interpretation is inadequate to explain other instances of heterosis; and he put forward in 1917 the familiar hypothesis which accounts for the decline of heterosis in generations subsequent to the F_1 on the basis of the segregation of many linked factors for size. Despite a criticism from Collins (1921) this hypothesis is still generally accepted. It conforms with the trend of ideas in modern genetics, since one can now assume that each factor influences many processes in the organism, and that the greater the number of 'favourable' dominants the organism carries, the more efficient is its metabolism likely to be. On Jones' hypothesis, and according to modern views on the action of genetic factors, the metabolic processes in a hybrid from dissimilar homozygous parents should be accelerated; in fact East has recently gone so far as to assert that this is so, although he adduces no evidence to support his assertion (1936, pp. 380, 389). There will be exceptions, of course, as when one of the parents in a cross carries a dominant inhibitory factor, but in the experience of breeders such exceptions are rare.

The most obvious manifestation of increased metabolic efficiency is greater growth rate; and accordingly size, and in particular height, has usually been taken as a criterion of heterosis. Now the final size of a plant is dependent upon three factors: the size of individual cells, the size of primordia, and the relative growth rate. The problem as to which of these three factors was affected in heterosis was not investigated until 1930, when the author published a short analysis of the physiological basis of heterosis in a hybrid of maize. The data given in this paper led to an unexpected conclusion; namely, that despite considerable heterosis there was no evidence of a greater relative growth rate in the hybrids. Growth rate was measured as increase in dry weight, and was therefore representative of the net product of the plant's metabolism. There was no increase in cell size in the hybrids, so far as a cursory examination showed, but the hybrids had considerably larger apical primordia in the embryos than their parents. Analysis showed that the size-heterosis was completely referable to the larger primordia of the embryos.

In a subsequent paper (1932) the experiment was repeated on other strains of maize with precisely the same result. Further work showed that the respiration rate of the hybrid was not increased over that of its parents, and that there was evidence of segregation of relative growth rates in the F_2 generation. Size-heterosis, in terms of the analogy used by V. H. Blackman (1919), was found to be due to a greater 'capital' in the embryo, and *not* to a greater 'rate of interest'. It was emphasized (Ashby, 1932, p. 1031) that this capital refers to size of apical primordia in the embryo and not to stored food or to size of seed, both of which are apparently not correlated with size-heterosis. Since this point has been overlooked (East, 1936; Lindstrom, 1935) it must be stressed here, as essential to an understanding of the data which follow.

In the earlier papers of this series it was explicitly stated that the importance of embryo size for heterosis was established only for the particular strains used in the experiments, and that 'one is not justified in extending these conclusions further, even to other varieties of maize' (Ashby, 1932, p. 1027). Nevertheless, Lindstrom (1935) and East (1936) have discussed the author's work as though it claimed to be a general solution of the problem of heterosis. It is rather a statement of what heterosis is *not*. The conclusions which can be drawn from the experiments already published are:

(i) that in the strains studied heterosis is accompanied neither by a greater relative growth rate nor by a greater respiration rate nor by a greater assimilation rate;

(ii) that the hybrids showing size-heterosis had larger primordia in their embryos, and that size-heterosis in these strains was primarily due to the maintenance of this initial advantage;

(iii) that as a consequence of the second conclusion the answer to the problem of size-heterosis in these cases must be sought between fertilization and the setting of seed.

So far as the writer is aware there are no data in the literature at variance with these conclusions. The data might be made to fit the hypothesis of Jones by assuming that both parents in the cross carried the same factors for the processes which are not accelerated in the hybrids, i.e. that in respect of factors controlling growth rate the parents were not dissimilar. Such an assumption would be possible if the conclusions stated above applied only in one or two exceptional instances; but to make the assumption in many instances would clearly be absurd. Before embarking on any modification of Jones' hypothesis it should be established that the present author's conclusions on size-heterosis in maize have a wider application. Accordingly heterosis in other plants has been examined, and this paper presents the first part of an analysis of heterosis in tomatoes.

MATERIAL AND METHODS

For convenience of analysis the life-cycle of the tomato may be divided into three phases: (i) from germination to the onset of flowering, a period which is defined for each variety by the number of nodes produced before the first inflorescence; (ii) subsequent growth during the flowering period, an ill-defined phase, for a tomato plant may produce many trusses of flowers before dying; (iii) growth of the embryo from the time of fertilization to the setting of seed. In the present paper data are advanced for the first part of the life-cycle only. In a subsequent communication the second part will be considered; and data are being collected in this laboratory for an analysis of growth in the third phase of the cycle.

The experimental procedure was to grow a population of two inbred parents and their F_1 offspring under uniform conditions, and to analyse the growth rate by periodic sampling of the plants. From the samples data were obtained as to height, wet weight, dry weight, leaf area, and morphological development. From the treatment of these data the proximal cause of hybrid vigour, so far as it affects size, was easily determined. The experiments were terminated after the first truss of flowers had opened. Six inbred strains of tomatoes have been tested in this way, and the data may conveniently be discussed under three experiments, as follows:

Experiment 1. 1934.

Parent 1, known to be homozygous for the following factors: *D*, *P*, *O*, *R*, *Y*, *b*; described as a tall strain.[1]

Parent 2, known to be homozygous for the following factors: *d*, *p*, *r*, *y*; described as a dwarf strain.

Hybrid, the F_1 generation from the cross of parent 1 by parent 2, parent 1 being the female parent.

Experiment 2. 1934.

Parent 1, known to be homozygous for the following factors: *d*, *p*, *o*, *r*, *y*, *s*, *A*; described as a dwarf strain.

Parent 2, known to be homozygous for the following factors: *D*, *P*, *O*, *R*, *Y*, *S*, *a*; described as a tall strain.

Hybrid, the F_1 generation from the cross of parent 1 by parent 2, parent 1 being the female parent.

Experiment 3. 1935.

Parent 1, an inbred strain of *Solanum racemigerum*, a tall plant, closely resembling the tomato, but with smaller fruits. Although its genetic constitution is not determined, the strain is apparently homozygous for *D*, *P*, *O*, *R*, *Y*, *S*; or for factors which are homologous to these.

Parent 2, the same strain as parent 1 in experiment 2.

Hybrid, the F_1 generation from the cross of parent 1 by parent 2, parent 1 being the female parent.

The procedure was the same in all three experiments. The three populations compared in each experiment were grown under good cultural conditions in a greenhouse. The only departures from ordinary cultural methods were that seeds were soaked twenty-four hours before sowing, were germinated in 10-inch pots, and were not transplanted at any time during the experiment. The plants were thinned to one per pot while still in the cotyledon stage, and the lateral branches were not pruned out as they frequently are in commercial houses.

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[1] I am indebted to Dr. F. W. Sansome for this and the following material. The characters for which the factorial symbols stand are described in his paper (1933) and by Macarthur (1926). They are: *d* = dwarf, as opposed to tall; *p* = peach surface of fruit, as opposed to shiny; *o* = oval fruit, as opposed to round; *r* = not red skin, as opposed to red skin; *y* = clear skin, as opposed to waxy; *s* = compound inflorescence, as opposed to simple; *a* and *b* = factors inhibiting anthocyanin production. The factors *d*, *p*, *o*, and *s* are in the same linkage group.

In order to take account of environmental differences within the greenhouse, it was divided for experiments 1 and 2 into four 'blocks'. Each block contained twelve pots of each of the three populations, i.e. thirty-six pots in all. The parent strains and their hybrids were arranged in random rows within each block. The procedure in sampling was as follows. Every pot in each block was numbered. Lots were drawn to decide which pots should be sampled; two of each strain were taken from each block, making a total of eight plants per strain in each sample. The variance between duplicates from each block formed the estimate of error. The samples were carefully removed from the soil, and their roots enclosed between gauze plates and washed in running water. The following observations were taken: height, number, and length of internodes, fresh weight and dry weight of leaves, shoots, and roots, leaf area, and remarks where necessary as to the growth of laterals and flowering. The weighing was carried out on spring balances similar to those described by Bolas and Melville (1933). For dry weights the plants were left for twenty hours in an oven at 100° C. Leaf areas were determined with the aid of a photo-electric cell, by a modification of a method described by Ashby and Oxley (1935). In addition to the data collected from the growth experiments observations were made on seed weight, embryo weight, &c.

In experiment 3 a slightly different procedure was adopted. The greenhouse space was divided into three blocks instead of four, and nine plants of each strain were harvested, three from each block. This was done in order to provide a more sensitive test of significance. Since the aim of the third experiment was to confirm the principal conclusions from the other two, dry weight and leaf number were the only data collected.

PRESENTATION OF DATA

Before one can draw conclusions with any confidence from the data it is necessary to subject them to statistical analysis. Since the details of this analysis are not of general interest, it has been put in an appendix to the paper, together with notes on the methods used. The statistical significance of results to be discussed in this section may be verified, therefore, by reference to the appropriate tables in the appendix.

The data are too extensive to be reproduced in full. In Table I is a summary of the results of the three experiments. Each entry under experiments 1 and 2 is the *mean* of eight samples from four blocks. Each entry under experiment 3 is the *mean* of nine samples from three blocks. The relevant data from Table I are discussed in the following paragraphs.

Dry weight.

Dry weight provides the most comprehensive summary of growth. Up to the time of appearance of the first inflorescence the relative growth rates are approximately constant, so that logarithms of dry weight plotted against time

TABLE I

EXPERIMENT 1	Parent 1.							Parent 2.							Parent 1 × Parent 2 (Hybrid).						
Days from sowing	10	17	24	31	38	45	52	10	17	24	31	38	45	52	10	17	24	31	38	45	52
Height (cm.)	2·2	3·7	6·9	16·0	24·0	28·3	38·0	1·6	2·8	5·4	13·0	20·4	25·1	34·0	2·6	5·2	9·8	18·0	26·8	33·9	41·0
Number of leaves	0	2·62	4·5	7·75	9·38	9·75	12·2	0	2·25	4·25	6·62	8·50	9·63	11·5	0·8	4·0	5·63	8·25	9·88	10·5	13·0
Leaf area (cm.^{2})	0	6·98	44·1	215·0	329·0	364·0	—	0	6·5	44·6	141·0	322·0	362·0	—	0	16·4	114·9	232·0	320·0	439·0	—
Fresh weight: stem & root	—	0·29	0·81	3·81	7·74	10·73	—	—	0·27	0·97	3·46	7·74	9·52	—	—	0·45	1·99	4·58	8·86	12·40	—
Fresh weight: leaves	—	0·17	1·16	5·63	9·50	12·23	—	—	0·17	1·27	4·58	9·68	11·41	—	—	0·44	3·09	6·40	10·68	14·93	—
Total fresh weight (gm.)	—	0·46	1·97	9·44	17·24	23·06	—	—	0·44	2·24	8·04	17·42	20·93	—	—	0·89	5·08	10·98	19·54	27·33	—
Dry weight: stem & root	—	0·021	0·055	0·31	0·80	1·29	—	—	0·019	0·062	0·31	0·82	1·29	—	—	0·029	0·12	0·40	1·03	1·57	—
Dry weight: leaves	—	0·016	0·100	0·71	1·58	2·13	—	—	0·017	0·109	0·71	1·27	1·63	—	—	0·041	0·27	0·86	1·77	2·44	—
Total dry weight (gm.)	—	0·037	0·155	1·02	2·38	3·42	—	—	0·036	0·171	1·01	2·09	2·92	—	—	0·070	0·39	1·26	2·80	4·01	—

EXPERIMENT 2	Parent 1.							Parent 2.							Parent 1 × Parent 2 (Hybrid).						
Days from sowing	10	17	24	31	38	45	52	10	17	24	31	38	45	52	10	17	24	31	38	45	52
Height (cm.)	1·99	3·2	5·6	11·1	16·2	20·3	25·0	2·4	3·7	7·8	15·5	18·5	24·6	39·0	2·2	4·3	8·1	16·4	23·4	28·6	42·0
Number of leaves	0	3·0	5·13	7·62	9·5	11·25	13·0	0	3·62	6·0	9·1	10·1	12·4	16	0	3·25	5·62	8·7	10·6	11·9	16·2
Leaf area (cm.^{2})	0	11·34	53·9	155·0	259·0	329·0	—	0	14·9	107·6	257·0	315·0	400·0	—	0	14·65	111·5	279·0	393·0	440·0	—
Fresh weight: stem & root	—	0·30	0·87	2·78	5·91	8·73	—	—	0·32	1·15	3·81	5·31	8·71	—	—	0·35	1·26	4·39	8·09	10·59	—
Fresh weight: leaves	—	0·26	1·45	4·71	8·04	10·98	—	—	0·35	2·63	7·44	9·09	13·59	—	—	0·35	2·73	8·68	12·22	15·00	—
Total fresh weight (gm.)	—	0·56	2·32	7·49	13·95	19·71	—	—	0·67	3·78	11·25	14·40	22·30	—	—	0·70	3·99	13·07	20·31	25·59	—
Dry weight: stem & root	—	0·027	0·063	0·24	0·66	1·25	—	—	0·023	0·084	0·36	0·56	1·25	—	—	0·027	0·089	0·39	0·97	1·53	—
Dry weight: leaves	—	0·024	0·131	0·56	1·12	1·53	—	—	0·033	0·23	0·95	1·44	2·28	—	—	0·038	0·24	1·10	2·11	2·65	—
Total dry weight (gm.)	—	0·051	0·194	0·80	1·78	2·78	—	—	0·056	0·314	1·31	2·00	3·53	—	—	0·065	0·329	1·49	3·08	4·18	—

EXPERIMENT 3	Parent 1.				Parent 2.				Parent 1 × Parent 2 (Hybrid).			
Days from sowing	10	20	30	40	10	20	30	40	10	20	30	40
Dry weight	0·009	0·036	0·542	3·04	0·004	0·026	0·455	2·71	0·015	0·08	1·262	4·96

Summary of data from the three experiments. Each entry under experiments 1 and 2 is the mean of 8 samples. Each entry under experiment 3 is the mean of 9 samples.

fall roughly along straight lines (Figs. 1, 2, and 3). Whether these logarithmic growth curves are linear or not is of no significance to the present problem; what matters is the relation between one curve and another in the same experiment.

Two items of information are required from the dry weight data: (i) did the hybrids show a significant amount of size-heterosis, and (ii) had the hybrids a greater relative growth rate than their parents? Analysis of variance of the values for dry weight provides this information. Tables for the complete analysis of dry weights in experiment 1 are given in the Appendix (p. 37), and the results for the three experiments are summarized in Table II.

TABLE II

Summary of Analysis of Variance of Logarithms of Dry Weight of Parent and Hybrid Strains in the Three Experiments. Significance (S) is assumed when the values of 'z' exceed the 1 per cent. points

Variance due to:	Expt. 1.	Expt. 2.	Expt. 3.
Strain of tomato . . .	S	S	S
Block of greenhouse . .	—	S	S
Time.	S	S	S
Interactions:			
strain × time . . .	S	—	S
block × time . . .	—	—	—
block × strain . . .	—	—	—
Triple interaction . .	S	—	—

The entries in this table will be considered in turn. The variance due to strain is significant in each experiment. This means that the three populations in each experiment differed in mean dry weight. It next has to be determined where these differences lie. From the analysis in the Appendix (pp. 38, 39) it is clear that the significant differences in dry weight are distributed as follows:

Experiment 1. No significant difference between the two parents. Hybrid heavier than both. Size-heterosis is therefore demonstrated.

Experiment 2. Parents differ in dry weight. Hybrid not significantly heavier than one parent. Therefore no significant heterosis is present.

Experiment 3. Difference between parents is significant. Hybrid is significantly heavier than both parents. Size-heterosis is therefore demonstrated.

The conclusion from this analysis is that the hybrids in experiments 1 and 3 show significant heterosis, measured as dry weight. In experiment 2 the hybrid showed no significant heterosis. These results are set out as graphs in Figs. 1, 2, and 3.

Turning now to the next entry in Table II it will be noticed that variance due to inequalities of environment is significant in the second and third experiments, and has been eliminated by the analysis. Variance due to time, the next entry in the table, is of course highly significant: it simply means that the plants are growing. The next entry, the *interaction* of strain and time, is important, for any differences in relative growth rate (efficiency index)

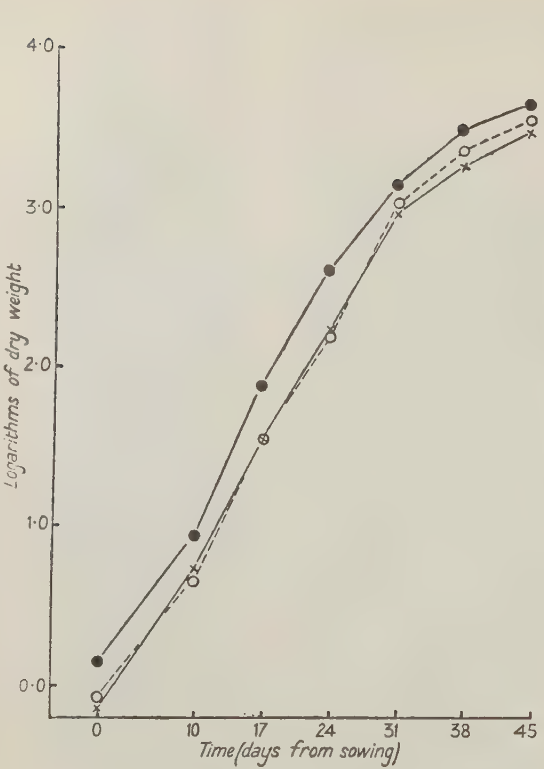

FIG. 1. Experiment 1. Logarithms of total dry weight plotted against time. Black circles hybrid, white circles parent 1, crosses parent 2. The entries at zero time are embryo weights.

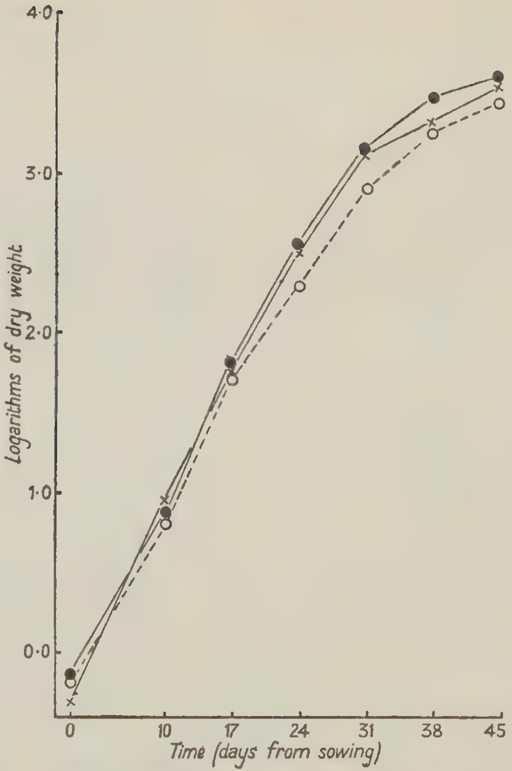

FIG. 2. Experiment 2. Logarithms of total dry weight plotted against time. Black circles hybrid, cross parent 1, white circles parent 2. The entries at zero time are the embryo weights.

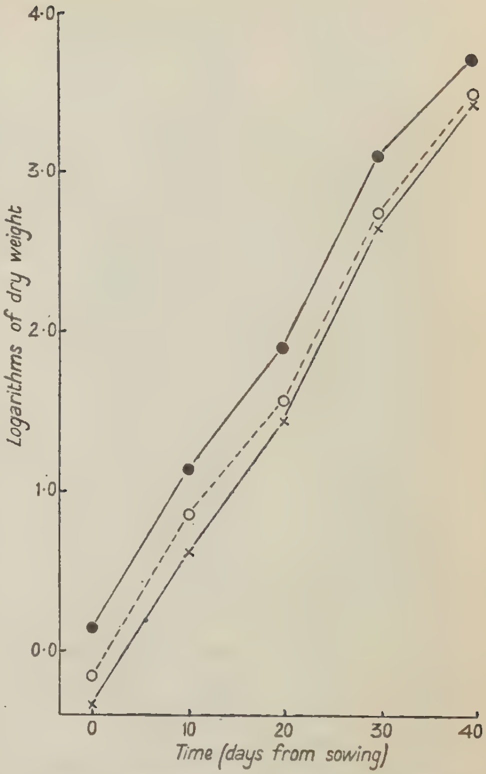

FIG. 3. Experiment 3. Logarithms of total dry weight plotted against time. Black circles hybrid, white circles parent 1, crosses parent 2. The entries at zero time are embryo weights.

will be recorded as a significant variance under this heading. In experiment 2 this interaction is not significant, which means that the three strains had the same efficiency index. In experiments 1 and 3 the interaction is significant, but further analysis of the interactions (Appendix, p. 39) shows that the significance lies in a difference in relative growth rate *between the two parents*.

TABLE III

Dry Weights of Ungerminated Embryos

	Weight (mg.).	S.E.	Log. wt.
Experiment 1			
Parent 1	0·808	0·00686	1·907
Parent 2	0·750	0·01092	1·875
Hybrid	1·330	0·01215	0·124
Experiment 2			
Parent 1	0·493	0·00207	1·692
Parent 2	0·780	0·01102	1·892
Hybrid	0·813	0·01512	1·910
Experiment 3			
Parent 1	0·663	0·01055	1·822
Parent 2	0·493	0·00207	1·692
Hybrid	1·363	0·01825	0·134

The relative growth rate of the hybrid does not differ significantly from that of one parent. The conclusion of the analysis is, therefore, that in no case is the relative growth rate of the hybrid higher than those of both its parents. Heterosis in the strains examined is not accompanied by higher efficiency index.

The fact that the greater weight of hybrids is not attributable to greater efficiency index is apparent from an inspection of Figs. 1 and 3. Differences in relative growth rate would appear as differences in slope of the logarithmic growth curves. Thus, if a hybrid were heavier *because* it was growing faster its growth curve would slope more steeply than those of its parents. In no case is there any evidence of a steeper slope in the hybrids.

Heterosis, in so far as it affects dry weight, is clearly not the result of greater relative growth rate during the period of the experiment, but is present in the first sampling ten days after sowing. In order to discover whether size heterosis was present before germination, seeds were soaked in formalin, and their embryos were dissected out, dried for one hour in vacuo at 70° C., and weighed according to a method described for Lemna (Su and Ashby, 1929). The results of the weighings are given in Table III, and the mean values, converted into logarithms, are entered on the ordinates at zero time in Figs. 1, 2, and 3. Where heterosis occurs it will be seen that the hybrid embryo is heavier, and by about the right proportion to account for the subsequent heterosis. It is noteworthy that in experiment 2, where there was no significant size-heterosis, the hybrid embryo was not significantly heavier than the embryo of parent 2. There is, therefore, the strongest evidence that size-heterosis up to the first inflorescence in these hybrids is due to the maintenance of an advantage given by an initially greater capital of primordia tissue. It must be emphasized again that the capital in question is capital in the biological sense, i.e. the number of primordial cells in the embryo.

It has to be determined whether the weight of primordia tissue is a measure of the number of cells in the primordium. This relation will hold only if there is no great difference in cell size between the strains. Measurements of cell size have been made on embryos of the strains used in experiments 1 and 3. Although increases in size of cells in the hybrid were recorded in experiment 1, they were not significant (Table IV).

TABLE IV[1]

Size of Cortical Cells in Radicles of Tomato Embryos, measured in Arbitrary Units

		Length.		Breadth.	
	no. of cells.	Mean.	S.E.	Mean.	S.E.
Experiment 1					
Parent 1 . . .	100	1·28	0·072	1·67	0·072
Parent 2 . . .	100	1·16	0·040	1·63	0·032
Hybrid	100	1·43	0·053	1·72	0·028
Experiment 3					
Parent 1 . . .	100	1·23	0·020	1·83	0·026
Parent 2 . . .	100	1·13	0·023	1·80	0·021
Hybrid	100	1·17	0·013	1·91	0·020

Reference to Figs. 1, 2, and 3 will show that the logarithms of embryo weight lie in some instances closely on the extrapolations of the growth curves. This striking conformity is probably of no significance, for there is an initial loss in dry weight on germination, and the embryo weights include cotyledons which are not really part of the primordial capital. What is undoubtedly important is that the relative amount of size-heterosis during the experiments is never significantly more than the relative amount of heterosis present in the embryos, and the final differences in size are referable entirely to initial differences in capital. The dependence of size-heterosis on embryo weight is strikingly illustrated in Fig. 2, from experiment 2. In this experiment there was no significant size-heterosis, and reference to Table III shows that there was no significant advantage in embryo size. Where there was a large initial advantage in embryo size, as in experiment 3, there was a correspondingly large amount of size-heterosis.

Height.

Dry weight, considered in the preceding section, is only one measure of growth; and perhaps the most striking manifestation of vigour in some hybrids is their height. Although height is patently unsatisfactory as a measure of size, it has frequently been chosen as the criterion of heterosis. On this account data on height were collected from experiments 1 and 2. The mean values are entered in Table I. Growth rate as height was exponential until the appearance of inflorescences; in Fig. 4 the logarithms of plant height are plotted against time for experiment 1. There is height-heterosis

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[1] The writer acknowledges the help of Mr. L. C. Luckwill, who made these measurements.

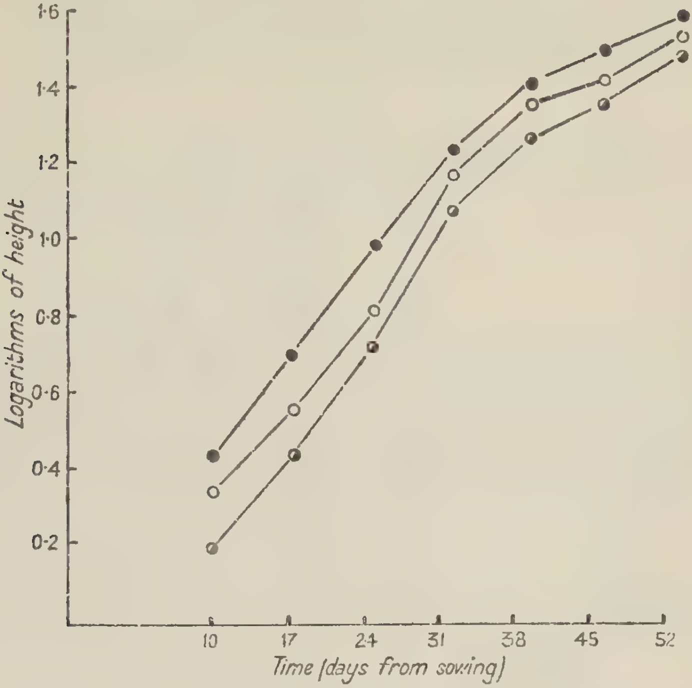

FIG. 4. Experiment 1. Logarithms of height plotted against time. Black circles hybrid, white circles parent 1, half-white circles parent 2.

TABLE V

Summary of Analysis of Variance of Logarithms of Heights of Parent and Hybrid Strains in Experiments 1 and 2. Significance (S) is assumed when values of 'z' exceed the 1 per cent. points

Variance due to:	Expt. 1.	Expt. 2.
Strain of tomato	S	S
Block of greenhouse	—	—
Time	S	S
Interactions:		
strain × time	—	—
block × time	—	—
block × strain	—	—
Triple interaction	—	—

apparent from the first sampling, and no evidence that the relative growth rate, as height, was any greater in the hybrid, despite this heterosis. From a statistical examination of these data it emerges that there *is* significant heterosis, but there is *no* significantly greater relative growth rate (Table V, and Appendix, p. 39). By analogy with the results for weight-heterosis, one might suggest that the proximal cause of height-heterosis lies in the possession by the hybrid embryo of slightly *longer* primordia, though since the embryo is mostly root and hypocotyl, one would not expect any close relation between embryo length and final height. Measurements of embryo length have been made for the strains used in experiment 1, and are presented in Table VI.

TABLE VI[1]

Lengths of Embryos of Strains used in Experiment 1. Values in millimetres

	Radicle Length.		Cotyledon Length.		
	Mean.	S.E.	Mean.	S.E.	Total.
Experiment 1					
Parent 1	3·63	0·040	4·08	0·090	7·71
Parent 2	2·97	0·045	3·71	0·148	6·68
Hybrid	3·76	0·039	4·51	0·079	8·27

The hybrid embryos are just significantly longer than those of parent 1 and considerably longer than those of parent 2. The evidence is not adequate to suggest that the height heterosis was already determined in the embryo, but there is no doubt there was some advantage in length, and that after the first sampling there was no greater relative growth rate on the part of the hybrid. Height-heterosis, therefore, is not due to a greater relative growth rate after the first sampling, though there may have been greater growth rate between germination and the first sampling.

TABLE VII

Mean Length of Lower Five Internodes. Data from Experiment 1
Each entry the mean of eight samples. Values in centimetres

Days from germination.	Parent 1.	Parent 2.	Hybrid.
31	3·02	2·66	3·04
38	3·24	2·91	3·18

It is worth mention at this point that the characters controlled by the factors *D* and *d* are apparently not concerned in heterosis. Plants homozygous for *d* are shorter than plants carrying *D*, even before germination (Table VI). From germination to the first inflorescence there is not much difference in height between tall and dwarf plants. The apparent difference in height is due partly to the fact that the angle of insertion of the petioles in *dd* plants is greater than in *Dd* or *DD* plants, so that the *dd* plants are more squat in appearance; there is some evidence that this angle of insertion is inherited in a particulate way. Dwarf plants have shorter internodes than tall plants, and internode length in the hybrid is apparently the same as in the taller parent (Table VII).

Data on the differences between the growth of tall and dwarf plants after flowering will be published in a subsequent paper.

A second point has to be raised here, namely, whether the greater height

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[1] The writer acknowledges the help of Mr. L. C. Luckwill, who made these measurements.

TABLE VIII

Cell Size, in Arbitrary Units, of Strains used in Experiment 3

		Parent 1.		Parent 2.		Hybrid.	
Tissue.	No. obs.	Mean.	S.E.	Mean.	S.E.	Mean.	S.E.
Upper epidermis 1st leaf .	100	30·5	1·03	10·0	0·88	20·5	2·61
Upper epidermis 2nd leaf .	200	34·6	2·38	12·3	0·35	24·2	0·83
Hypocotyl epidermis . .	150	21·3	1·13	16·0	0·42	19·7	1·51

is due to greater cell number or to bigger cells. As Sinnott and Dunn (1935) and Houghtaling (1935) have pointed out, the greater size of an organ is due sometimes to one of these factors and sometimes to the other. In any analysis of heterosis it is necessary to distinguish between them. This is a far more difficult matter than is sometimes thought, for Sinnott has shown that when one fruit is bigger than another the greater size may be due to more cell division in one tissue, and to greater cell enlargement in another! Any comparison of cell size, such as that presented in Table VIII, is incomplete as evidence unless all the tissues of the plant are measured. The measurements in Table VIII do show, however, that in the experiment which showed the maximum heterosis there is no indication of greater cell size in the organs measured, and that the inheritance of cell size in the leaves of the hybrid is intermediate.

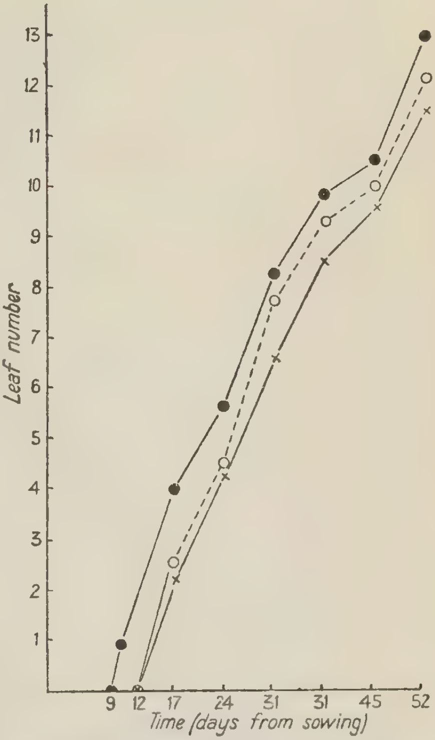

FIG. 5. Experiment 1. Leaf number plotted against time. Black circles hybrid, white circles parent 1, crosses parent 2. The entries on the abscissa at 9 and 12 days respectively mark the latest days on which less than 20 per cent. of the plants had unfolded their first leaf.

Leaf number.

In Fig. 5 the leaf numbers of the three strains in experiment 1 are plotted against time, and in Fig. 6 are the leaf numbers from experiment 2. The data from experiment 1 in particular bring out a further feature of heterosis: the hybrid has more leaves than either parent, but the *rate of leaf production is exactly the same.* In experiment 2, where there was less heterosis, the advantage in leaf number is very small, and the leaves unfolded at the same time in all strains. The phenomenon is easily explained by reference to the abscissa of the zero number of leaves in Fig. 5, on which are plotted the latest days on which not more than 20 per cent. of the first leaves had expanded, i.e. reached a length of more than 1 cm. It is seen that the whole advantage in leaf number in the hybrid can be referred to the fact that its first leaf unfolded about three days earlier than the leaves of its parents. This precocity of leaf production must not be confused with rate of germination. Attempts to relate the rate of germination of parents and hybrids give inconsistent results, partly because it is difficult to germinate a large population of seeds under standard conditions.

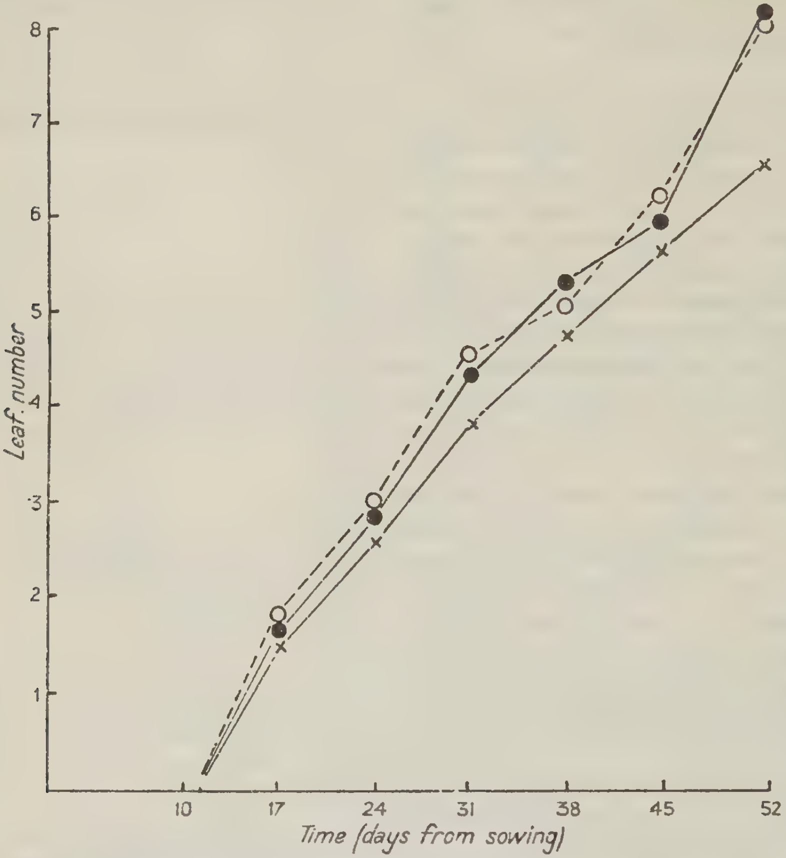

Fig. 6. Experiment 2. Leaf number plotted against time. Black circles hybrid, white circles parent 2, crosses parent 1. Leaves of all three strains unfolded about the 12th day.

Total leaf area.

In Fig. 7 the logarithms of total leaf area from experiment 1 are plotted against time. The same relationship appears as in the preceding measures of growth. Beside height and dry weight and leaf number, heterosis is expressed as increased leaf area, but this heterosis is not accompanied by a greater relative growth rate of area. The full amount of area-heterosis is present in the first sampling. The hybrid has a greater leaf area partly because its leaves appeared earlier than the leaves on the parents; they are therefore at a later stage of development, and there are more of them.

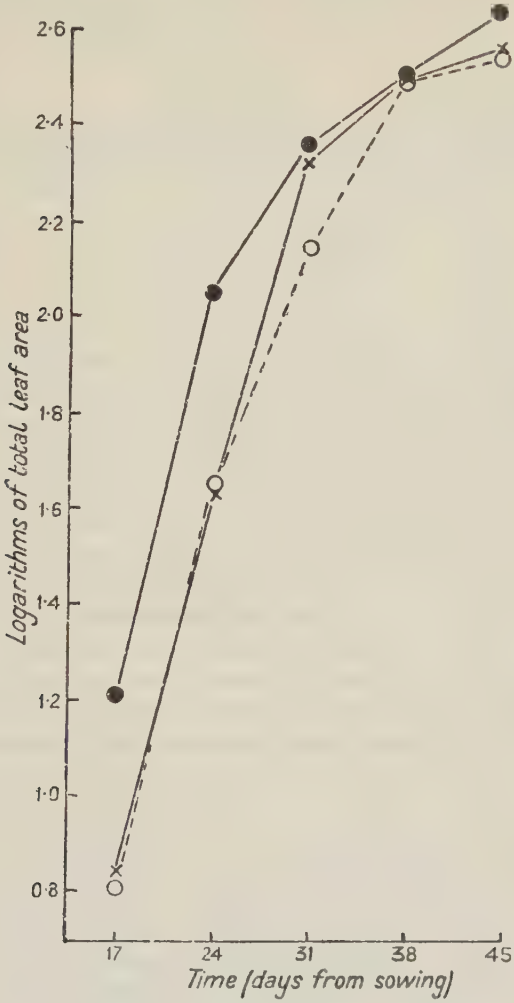

FIG. 7. Experiment 1. Logarithms of total leaf area plotted against time.

Area per leaf.

The relative growth rate of individual leaves is summarized for experiment 1 by Fig. 8. There is again no evidence of greater relative growth rate in the hybrid, despite the fact that the average area per leaf was greater until the onset of flowering. Heterosis, in so far as it affects leaf area, is attributable to the fact that the hybrid leaves are at a later stage of development than those on the parents (vide Fig. 5) and, as will subsequently be shown, to the fact that the leaf initials in the hybrid are bigger. Sinnott (1921) demonstrated fifteen years ago that the size of an organ was closely correlated with the size of the initial from which it grew, and the larger leaves of the hybrid illustrate Sinnott's preliminary thesis, for anatomic studies show that they grow from initials which are larger than those of the parents.

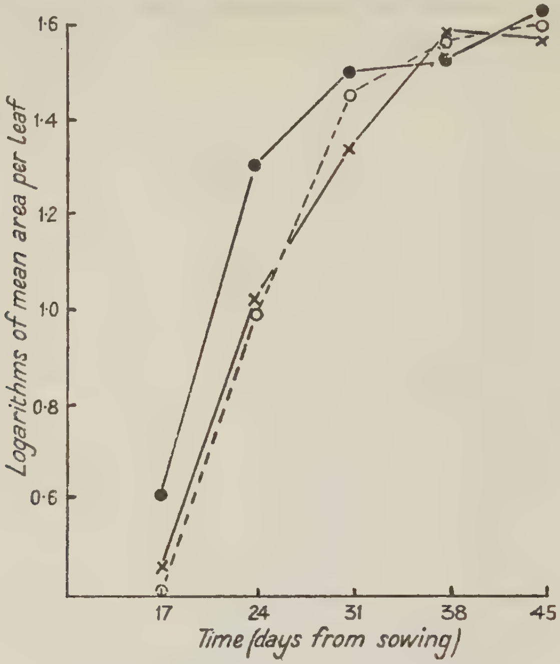

FIG. 8. Experiment 1. Logarithms of mean area per leaf plotted against time.

Rate of photosynthesis.

From the data for dry weight and leaf area it is possible to calculate the net assimilation rate of the various strains. In accordance with the conventional view of hybrid vigour one might expect to find a higher rate of photosynthesis in the hybrid, when data are taken over the growth season as a whole. Since in the early part of the life-cycle both dry weight and leaf area are increasing exponentially the mean rate of photosynthesis is: $\frac{W_t - W_0}{\bar{A}}$, where $\bar{A}$ is the mean area, and is $A_t - A_0/\log_e A_t - \log_e A_0$.[1] The assimilation

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[1] The formula for the mean leaf area is frequently used in plant physiology without its derivation being given. Since the formula is not self-evident, it may be convenient to give its derivation here.

The mean area between times t_1 and t_0 is

$$\frac{1}{t}\int_{t_0}^{t_1} A\,dt = \frac{1}{t}\int_{t_0}^{t_1} e^{kt}\,dt = \frac{1}{t}\left(\frac{1}{k}\,.\,e^{kt}\right)^{t_1} = \frac{e^{kt_1}-e^{kt_0}}{kt_1-kt_0} = \frac{A_{t_1}-A_{t_0}}{\log_e A_{t_1}-\log_e A_{t_0}}.$$

rates, expressed as mg. CO_2 per dm.^{2} per day are given in Table IX. The results of an analysis of variance on these data (Appendix, p. 40) are that no significant difference between the assimilation rates exists. It may be concluded, therefore, that the assimilation rate of the hybrid on a basis of leaf area is no greater than that of its parents.

TABLE IX

Assimilation Rates of Strains of Tomatoes used in Experiments 1 and 2, expressed as mg. CO_2 per dm.^{2} per day. Each entry is the mean of four observations

Experiment 1.		Experiment 2.	
Parent 1	168·1	Parent 1	146·5
Parent 2	177.8	Parent 2	150.2
Hybrid	153·0	Hybrid	162·0

SUMMARY OF DATA

It is evident in the first place that size-heterosis in the cross between tall and dwarf strains of tomatoes is not dependent on the factor for tallness. It is also evident that the hybrid, despite its significant extra vigour, does not exceed its parents in the following respects: (i) relative growth rate measured as dry weight increase; (ii) relative growth rate measured as increase in height; (iii) rate of production of new nodes and leaves; (iv) rate of increase of total leaf area; (v) rate of increase of area per leaf; (vi) cell size, as measured in the upper epidermis of the first and second leaves and the hypocotyl; (vii) rate of photosynthesis, measured as increase in dry weight per unit leaf area.

Despite these facts, the hybrid, in comparison with its parents, has greater wet and dry weight, greater height, more leaves, greater leaf area, and a greater total assimilation rate. In addition, the hybrids which showed significant heterosis had significantly larger primordia in the embryo, measured as length, breadth, and weight, than their parents had; while the hybrid which showed no significant heterosis (experiment 2) had an embryo which was not significantly bigger than that of one of its parents.

To explain these phenomena the following facts have been adduced:

(i) The greater dry weight in hybrids showing heterosis is due to the maintenance of an initial advantage in embryo weight. This is most probably indicative of a greater number of primordial cells, since the size of the cells is no greater in hybrids than in their parents.

(ii) The greater height is not due to a higher relative growth rate during the period of sampling. It is attributable partly to an initially greater length of primordium in the embryo, and *possibly* also to a more rapid elongation in the early stages of germination, though no data have been collected which demonstrate this.

(iii) The greater leaf number in hybrids is due entirely to the fact that the first leaf in the hybrid unfolds slightly earlier than the first leaves of either of its parents.

(iv) The greater leaf area is due partly to the fact that more leaves are unfolded, and partly to the fact that each leaf is at a later stage of development relative to the corresponding leaves on the parent plants.

(v) The greater gross assimilation rate is also due to the fact that the mean leaf area is greater. There is no evidence of higher photosynthetic activity on a basis of leaf area.

These conclusions apply to the strains of tomatoes examined in the foregoing experiments. They do confirm, however, and considerably extend, the results obtained with maize, and published in the earlier papers of this series.

Discussion

It is desirable to defer a general discussion of the physiology of heterosis until publication of work on the behaviour of hybrids during senescence and during embryo development. Work on both these phases is being carried out in this laboratory, and a paper on heterosis during senescence is already in preparation. Discussion at this stage will be confined, therefore, to the bearing of the present data on the problem of heterosis, and to comment on some recent publications which discuss the author's earlier work. These publications are of two kinds: those embodying new data on heterosis, and those which are polemical rather than scientific.

In the first category come papers by Passmore (1934), Engledow and Pal (1934), and Lindstrom (1935). Passmore published a short note on heterosis in *Cucurbita pepo*, in which she found that reciprocal crosses differed in seed weight, and that their subsequent heterosis (measured as dry weight, leaf area, and leaf number) was at first correlated with seed weights. At the end of the experiment, however, the reciprocal crosses had grown to the same size. She concluded that, although embryo size may determine differential heterosis at the beginning of the life-cycle, the duration of growth of two reciprocal crosses may differ, so that at the end they show the same amount of heterosis. Passmore's data support this conclusion, though, since she has only one estimate of dry weight in the life-cycle, it is impossible to follow the course of growth of her plants. The assumption she has made that seed weight is a measure of embryo weight still leaves some doubt as to whether seed weight is a measure of primordia size, for most of the embryo in Cucurbita is cotyledon. Her contribution is interesting chiefly as a preliminary analysis of the behaviour of hybrids during later periods of growth.

A contribution to the problem of heterosis in wheat has been made by Engledow and Pal (1934). They conclude that, 'Ashby's explanation of hybrid vigour in maize F_1's as resulting solely from the greater embryo weight of crossed grain, was inapplicable to wheat.' It is to be expected, of course, that the cause of heterosis will be different for different plants, but for this particular case Engledow and Pal do not publish evidence to support their conclusion. They state (p. 398) that the amount of vigour they obtained is small, and the results 'cannot be pressed to a critical analysis'. If this is so, it is not surprising that they could find no increase in embryo size in their hybrids where there would be even less chance of detecting significant differences. The material Engledow and Pal used is apparently unsuitable for an analysis of heterosis, since it is present in too small a degree.

Lindstrom (1935) has published a so-called 'test of the Ashby hypothesis'. Lindstrom's test has been discussed elsewhere (Ashby, 1936), but it needs mention here. He sets out to demonstrate that primordia size in the embryo is not the determining factor in size-heterosis, but that the relative growth rate of the hybrid is greater. He does not, however, take observations either of embryo size or relative growth rate, but reduces the 'capital' of the growing plants by cutting back the leaves above the growing point. He finds that despite the removal of 80 per cent. of the plant, as leaves, the hybrids exceed their parents in weight and yield at the end of the growth season.

Such an experiment is very interesting physiologically but is, of course, irrelevant to the present problem. One cannot regard any organ of the plant indiscriminately as 'capital'. So far as potentiality for cell division is concerned the only capital in the plant lies in the primordia. The effect of removing leaves is clearly quite different from the effect of removing buds or inflorescences. There is in fact evidence that removal of the photosynthetic organs will stimulate a plant to vegetative activity, whereas removal of the growing points would have the opposite effect. Unfortunately, therefore, Lindstrom's experiment throws no light on the cause of hybrid vigour and supplies no evidence, implicit or explicit, of heterosis being due to a greater relative growth rate of the hybrid.

One further point in Lindstrom's paper requires mention. He states (p. 320) that the inconsistency between his results and those of the present writer 'may rest on the fact that Ashby's data were gathered from the grand period of growth . . . whereas the present experiments involved the entire growth of the plants. . . . It may be that the vigorous F_1 hybrid makes its greatest relative growth . . . at the later stages of its history.'

Inspection of the graphs in the author's earlier work and in the present paper shows that maximal hybrid vigour was already present at the first sampling. Now manifestation of heterosis in the senescent part of the plant's life obviously cannot be invoked as the cause of heterosis at the beginning. The writer is of opinion that no inconsistency exists between Lindstrom's work and his own since the data collected concern different processes.

In the report of the Chief of the United States Bureau of Plant Industry for 1935 (Richey, 1935) there are a couple of paragraphs in which it is stated that the author's work has been repeated and confirmed, except that evidence has been obtained of a 'significantly higher growth rate of the hybrid plants for the first two weeks after germination'. After that time no difference in rate could be detected. If this occurred in the writer's first experiments it would have been missed, for the first sample was taken twenty days after sowing, but in later experiments there has been no evidence of a higher initial growth rate. When the data are published it will be interesting to see what proportion of the heterosis is attributable to the higher efficiency index in the first fourteen days, and what proportion to the larger embryo. It is significant that the Bureau of Plant Industry has found that 'germ weight, or increase in germ weight over that of the parent may be of value in selecting desirable combinations of selfed strains'. In the author's opinion it is unlikely that embryo weight will be closely correlated with fruit yield, though it may under some circumstances be correlated with yield of vegetative parts.

Finally, brief mention must be made of a pronouncement by East (1935) which states *inter alia* that: 'Ashby's physiological theory of heterosis is unsound in all its essential features.' No new data are adduced in support of this statement, and the instances from earlier work on which East's assertion rests are irrelevant or misinterpreted. For example, he gives several cases where the occurrence of heterosis is not accompanied by bigger *seeds*, treating seed weight as a measure of embryo weight; although East has himself published data (East and Hayes, 1911, Table xv, p. 568) showing that strains of maize with the same 'seed' weight (grain weight) may have embryos differing in weight by thirty per cent. He asserts that 'Heterosis is ordinarily manifested by a rise in the efficiency index, in the sense with which Ashby used the term', and that it is 'a genetic effect on the organism as a whole', and in support of both statements he cites the author's data on maize, *which lead to the opposite conclusion*. East also credits the author with a 'physiological theory of heterosis' despite the fact that the author had emphasized that his explanation of heterosis applied only to the particular strains of maize he had studied. The more constructive part of East's paper discusses the occurrence of heterosis among amphidiploids, which, in East's opinion, supports Jones' hypothesis that heterosis is determined by a large number of genetic factors. East's view of heterosis may be summed up by the following two quotations: Heterozygosis 'effects a result comparable to favourable external conditions' (1916); and 'the effect of hybrid vigour is comparable to the effect on a plant of the addition of a balanced fertilizer to the soil' (1936, p. 389). This implies an increase in efficiency index in the hybrid; *all the evidence published up to the present is, however, at variance with these two statements.*

The conclusions published in earlier papers of this series were advanced to cover the phenomena of heterosis in four strains of maize. It has now been established that the same conclusions apply to six strains of tomatoes. This suggests that the dependence of heterosis on embryo size is of wider application than was originally thought. It is worth while, therefore, to examine the causes of heterosis in other organisms, in so far as they can be elucidated from the data of other workers. In the examples which follow, it must be emphasized that the writer's conclusions are not supported by any tests of significance. They are not valid proofs, and must be taken merely as likely and reasonable assumptions.

(i) *Data of Jones on growth in height of maize* (Jones, 1918, Fig. III, p. 58), *quoted by East and Jones* (1919).

On page 152 of East and Jones' excellent summary of heterosis up to the year 1919 there is a diagram illustrating height heterosis in maize. The data are reproduced in Fig. 8, without the growth curve of the F_2 generation, which does not bear on the present problem. There is considerable height-heterosis: the F_1 was nearly 3 feet higher than the taller parent, and about 5 feet higher than the shorter parent, after ninety days' growth. East and Jones accordingly assumed that the hybrid was growing faster than its parents. But if the logarithms of height are plotted against time the two parents and their hybrid are seen to be growing at about the same relative rate (Fig. 10). The advantage in height of the hybrid was fully present *before the experiment began*, and during the period of sampling there was no difference in relative growth rate. The mean relative growth rates for the whole period are:

Parent P (1–7) = 0·0188
Parent P (1–9) = 0·0190
Hybrid = 0·0192

Without a statistical analysis it is impossible to say that the difference in relative growth rate between the hybrid and parent 1–9, i.e. 0·0002 or 1·04 per cent. of the mean, is not significant, but since the variance of homozygous populations of maize is about 10 per cent., one can be fairly confident that there is no significant difference. At any rate it does not account for the heterosis observed, which must be attributed either to a larger initial embryo size or to a higher growth rate in the first 40 days of growth.

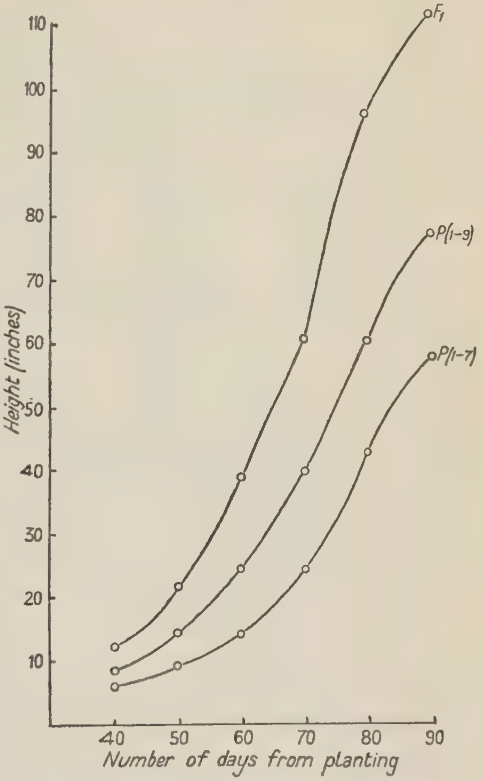

FIG. 9. Heights of three strains of maize (P 1–9, P 1–7, and the F_1 between them) from 40th to the 90th day of growth. Data from Jones (1918). Note the considerable height-heterosis.

(ii) *Data of Castle on growth in weight of guinea-pigs* (1916).

In his work on the genetics of size in guinea-pig crosses Castle published growth curves of weight for inbred guinea-pigs, wild cavies, and the first generation cross between them. The diagram (1916, Fig. 2) is reproduced by East and Jones (1919, Fig. 35) as an example of heterosis. Male and female animals were dealt with separately. The hybrids were heavier at birth, and showed heterosis throughout their lives. Thus, after 280 days the mean weights of male animals were as follows: cavies 394 gm., guinea-pigs 745 gm., hybrids 845 gm.

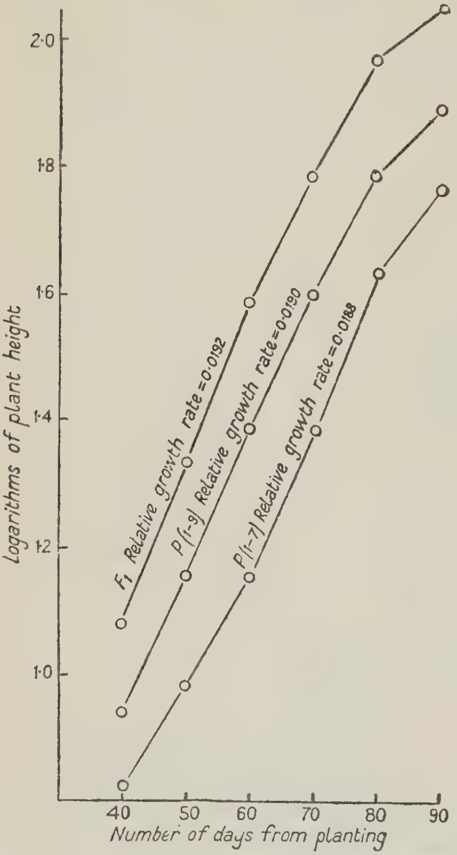

Fig. 10. Logarithms of heights of three strains of maize, data from Figure 9. Note that despite heterosis there is no difference in relative growth rate. Calculated from data of Jones (1918).

In Fig. 11 the logarithms of weight are plotted against time for the male animals. The relative growth rate fell throughout the life-history, but the ratio of weights between the hybrid and inbred guinea-pigs remained approximately the same throughout growth. For example, the hybrids were 30 per cent. heavier after 8 days, and they were still only 27 per cent. heavier after 160 days. In Table X the relative growth rates for successive periods of growth are given. Despite the obvious heterosis, the relative growth rate is no higher in the hybrid, except over the first period, where it is 4·8 per cent. higher. The greater size is clearly due to the maintenance of an initial advantage in birth weight.

(iii) *Data of Roberts and Laibile on growth in weight of pigs* (1925).

Weight measurements of inbred and hybrid pigs, published by Roberts and Laibile (1925), have been used by East as evidence that the author's conclusions for heterosis in maize are fallacious (East, 1936, p. 379). It is instructive, therefore, to examine their data in the light of the conclusions reached in this paper.

A Duroc Jersey sow was mated first to a Poland China boar, and twelve hours later to a Duroc boar. Progeny from the two boars may be distinguished by their colour. Six pure Durocs were born (four female and two male) and four hybrids (two female and two male). Since no male Durocs survived attention must be confined to the females in the litter. The hybrids showed weight-heterosis, even at birth. After six months the surviving animals were weighed again. The hybrids were now over 40 lb. heavier than the inbred animals. The data, together with the author's calculation from them, are given in Table XI.

It is to be noticed that the hybrid was 25 per cent. heavier at birth, and 23 per cent. heavier after six months. Size-heterosis is again referred entirely to the maintenance of an initial advantage in birth weight. There is no evidence of a greater efficiency index accompanying heterosis. Since one fertilization occurred twelve hours before the other, this may not, of course, be a case of heterosis at all, but of a longer growth period (see p. 25). It seems that in the examples quoted, as in the writer's own work, the effect of heterosis on size is determined before birth (in mammals) or before germination or the first sampling (in plants).

Table X

Relative Growth Rates of Male Inbred Cavies and Guinea-pigs, and the F_1 *Hybrid between them. Data calculated from Castle* (1916)

Time-period.	*Cavia cutleri.*	Guinea-pig.	Hybrid.
8th–40th day	0·0120	0·0100	0·0126
40th–60th „	0·0062	0·0079	0·0067
60th–80th „	0·0049	0·0059	0·0044
80th–160th „	0·0017	0·0024	0·0019
160th–200th „	0·0004	0·0015	0·0007
200th–360th „	0·0003	0·0006	0·0004
Mean	0·0037	0·0041	0·0039

Table XI

Data on Weights of Female Progeny from a Double Mating of Duroc Jersey Sow with (i) *Poland China Boar, and* (ii) *Duroc Boar. Primary data from Roberts and Laibile* (1925). *Values in pounds*

	Duroc × Duroc.	Duroc × Poland.	Ratio.
Birth	3·15 (mean of 3)	3·95 (mean of 2)	1·25
After 6 months	185·5 (mean of 2)	228·0 (mean of 2)	1·23
Efficiency index	2·770	2·761	

The discussion may be summed up in the following terms. Evidence from maize (data from Jones and the writer), from tomatoes, from Cucurbita (for the early part of the life-cycle), from guinea-pigs, and from pigs, supports the suggestion that size-heterosis is determined by embryo size (i.e. the primordial part of the embryo in seeds, and the weight at birth of mammals). In each of these cases there was significant size-heterosis without any significant increase in relative growth rate after the first sampling, so far as can be told from the data; and in each case there is evidence that the whole advantage in size was present before the experiment began. The conclusion that heterosis acts through '*capital*' and not '*rate*' *of interest* may therefore be extended to cover several plants and animals. It must still be emphasized that size-heterosis *may* operate in other ways, but the evidence so far accumulated points to its operating through increase of capital. One cannot generalize further from so few data; and it must be emphasized, too, that these conclusions refer only to size-heterosis. Other manifestations which accompany heterosis, e.g. better germination, viability, greater fecundity in animals, and so on, can obviously have nothing to do with embryo size, and their cause must be sought elsewhere.

The evidence, therefore, points to an action of heterosis through 'initial size' and not through metabolic rates during subsequent growth. Even if this principle turns out to be widely applicable it is, of course, no solution of the problem of heterosis: it merely refers the problem back to the beginning of the life-cycle. Two issues are raised: (i) if metabolic processes are controlled by genetic factors, and if Jones' hypothesis is correct, why is it that relative growth rate, which is the resultant of the organism's metabolism, is not increased when heterosis is present? (ii) What processes bring about the increased birth weight of animal and embryo weight of plant hybrids, which determine heterosis in the examples cited in this paper?

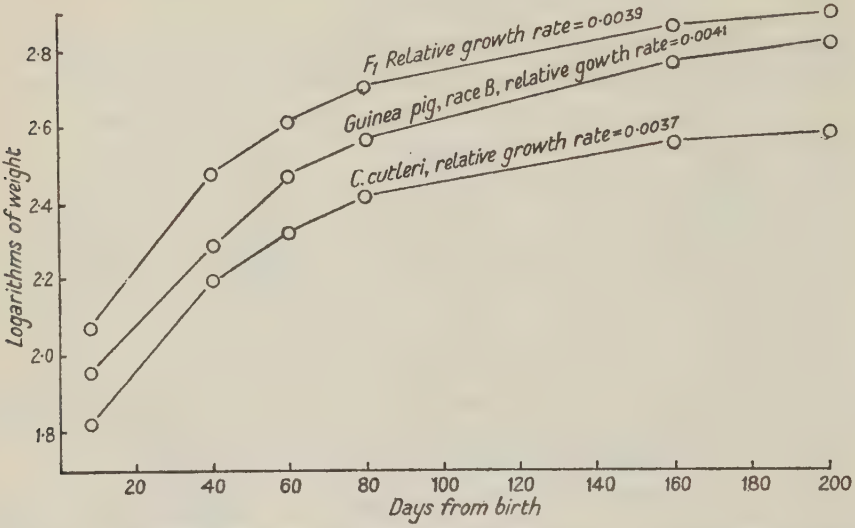

FIG. 11. Logarithms of weight of guinea-pigs, cavies, and the F_1 between them, plotted against time. There is no difference in relative growth rate, despite heterosis. Data from Castle (1916).

The writer is unable to answer the first question, and to obtain an answer to the second may need a refinement of technique. Bigger birth weight or embryo weight could be attained in three ways: (i) earlier division of the zygote, (ii) greater relative growth rate of embryo, (iii) embryo growth rate maintained over a longer period. It is easy to see that if the hybrid zygote divided even half an hour before the zygotes of parental types all the subsequent heterosis could follow, and a difference of this order would be difficult to detect. There is a little evidence from animals that size differences are determined very soon after fertilization. Gregory and Castle (1931) have shown that size differences in rabbits are established 40 hours after mating, although they were not present 30¼ hours after mating. The writer can find in the literature no evidence of this sort for plants, but an attempt is being made in this laboratory to collect data on the subject.

SUMMARY

Three experiments are described in which two inbred parent strains of tomatoes and the F_1 cross between them were grown under uniform conditions. Data were collected for height, leaf number, leaf area, wet and dry weight of different parts, and for cell size. These data were subjected to a statistical analysis.

Data were collected as to weight, dimensions, and cell size of the embryos of the six strains used for experimentation.

Significant size-heterosis occurred in two of the experiments.

The hybrids showing heterosis, compared with their parents, had greater wet and dry weight, greater height, more leaves, greater leaf area, and a greater absolute assimilation rate.

Despite this significant heterosis the hybrids did *not* exceed their parents in the following respects: (i) relative growth rate from the tenth to the fifty-second day measured as dry weight increase; (ii) relative growth rate over the same period measured as increase in height; (iii) rate of production of new nodes and leaves; (iv) rate of increase of total leaf area and of area per leaf; (v) cell size, as measured in the upper epidermis of the first and second leaves, and in the hypocotyl; (vi) rate of photosynthesis, measured as increase in dry weight per unit leaf area.

The hybrids showing size-heterosis were found to have embryos which were heavier and longer than the embryos of their parents. Evidence is adduced to show that size-heterosis in these hybrids is due to the maintenance of the initial advantage given by this embryo size. Cell size was no greater in hybrid embryos.

In the experiment where there was no significant heterosis it was found that the hybrid embryo had no initial advantage in size of the embryo over one of its parents.

The greater leaf number in hybrids showing heterosis is due to the fact that the first leaf in the hybrid unfolds slightly earlier than the first leaves of either of its parents. Where there was no advantage in leaf number there was no earlier unfolding of the first leaf.

The evidence adduced in earlier papers of this series was that in certain strains of maize size-heterosis operates through an increase in the biological capital in the embryo and not through a greater relative growth rate. In this paper the same conclusion is drawn for tomatoes.

In the discussion it is shown that data collected on heterosis by Jones (for maize), by Castle (for guinea-pigs), and by Roberts and Laibile (for pigs) all conform to the hypothesis that 'initial size' determines size-heterosis, and that there is no significant increase in relative growth rate of hybrids over their

parents. By 'initial size' is meant birth weight of mammals and embryo weight (or height at the first sampling) in plants.

It is emphasized that heterosis *may* operate in other organisms by increasing the relative growth rate, but no data which demonstrate this have yet been published.

It is pointed out that the dependence of heterosis on embryo size, even if it proves to be a common phenomenon, does not solve the problem of what heterosis is, but merely refers it to an earlier stage of the life-cycle.

In two of the experiments the parental strains differed in relative growth rate, and the hybrids inherited the growth rate of one parent in the manner of a complete dominant.

ACKNOWLEDGEMENTS

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APPENDIX

Statistical Analysis of the Results

1. *Method.* It is customary to analyse logarithmic growth curves, if they are straight lines, by calculating regression coefficients with their corresponding standard errors. For some of the data in the present paper, where the logarithmic curves are not strictly linear, this would involve fitting curved regression lines, which is a very laborious process. Accordingly a method using the analysis of variance has been employed (Fisher, 1928). Logarithms of the measure of growth (e.g. weight or height) are set out in a table (as in Table XII). The total variance among the entries in the table can be analysed into seven parts, as detailed in Table XIII. Where necessary the sums of squares were partitioned among the available degrees of freedom by choosing pairs of orthogonal expressions and substituting the appropriate values. A significant strain variance, if it lies between the hybrid and its parents, demonstrates heterosis. A significant interaction of strain and time demonstrates a difference in relative growth rate. These are the items required from the data. The tables of analysis are too extensive to be reproduced in full, but enough details are given to enable the calculations of experiment 1 to be repeated.

2. *Dry weight data.* Experiment 1. Table XII contains the logarithms of all observations on total dry weight in experiment 1. Table XIII contains an analysis of variance of these observations. Variances due to time and to strain are significant; so also is variance due to the interaction of time and

TABLE XII

Logarithms of Total Dry Weight for all Samples in Experiment 1. The variance due to differences of environment in the greenhouse is extracted by estimating the variance between the four blocks. The two samples taken from each block (1 and 2) provide the estimate of error. There are 143 degrees of freedom in the table, which are partitioned in Table XIII

Strain.	*Parent 1*								*Parent 2*								*Hybrid*							
Block.	I		II		III		IV		I		II		III		IV		I		II		III		IV	
Sample.	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Days:																								
10	0·361	0·447	0·763	0·820	0·724	0·361	0·792	0·708	0·748	0·763	0·845	0·672	0·881	0·785	0·580	0·663	0·763	0·820	1·004	0·833	1·017	0·964	1·004	0·748
17	1·672	1·591	1·380	1·531	1·643	1·362	1·415	1·748	1·279	1·732	1·398	1·591	1·342	1·799	1·580	1·398	1·869	1·623	2·100	1·881	1·690	1·851	1·857	1·732
24	2·053	2·143	2·380	2·111	2·228	2·140	2·182	2·201	2·117	2·290	2·452	2·097	2·170	2·236	2·243	2·164	2·602	2·653	2·580	2·556	2·663	2·643	2·602	2·415
31	2·940	3·121	2·982	3·093	3·013	3·117	2·851	2·833	2·857	3·004	2·839	2·806	2·964	2·886	2·935	2·732	2·919	3·149	3·127	3·021	3·274	3·045	3·057	3·104
38	3·373	3·267	3·439	3·378	3·427	3·367	3·378	3·358	3·230	3·390	3·487	3·167	3·367	3·358	3·236	3·217	3·535	3·479	3·444	3·497	3·444	3·344	3·465	3·318
45	3·568	3·591	3·447	3·580	3·531	3·431	3·462	3·613	3·602	3·477	3·204	3·447	3·556	3·519	3·362	3·431	3·531	3·556	3·633	3·623	3·663	3·491	3·602	3·690
Totals Blocks	28·127		28·904		28·344		28·541		28·489		28·005		28·863		27·541		30·499		31·299		31·089		30·594	
Totals for Strains	113·916								112·898								123·481							

strain. The totals of the logarithms of weight for the parent strains are 113·916 and 112·898, i.e. they differ only by 1·018. Partition of the variance of strain weight shows that significance lies between the hybrid and its parents: heterosis is demonstrated.

TABLE XIII

Variance due to	d.f.	Sums of sq.	Var.	'z'.	1% points.
Strains	2	1.420315	*0·710157*	1·856	0·5522
Blocks	3	0·053897	0·017966	0·113	0·6651
Time	5	140·752739	*28·150548*	—	—
Strain × time	10	0·540636	*0·054063*	0·6341	0·490
Block × time	15	0·233949	0·007798	—	—
Block × strain	6	0·093220	0·015536	—	—
Triple interaction	30	0·320596	0·010686	—	—
Error	72	1·021848	0·014192	—	—
Total	143	144·437200	—	—	—

The interaction of time and strain also is significant. This means that there was a significant difference in relative growth rate. The sums of squares of deviations in this interaction may be partitioned as follows:

Between parents 1 and 2	1.2231
Between parent 1 and hybrid	0·2055
Between parent 2 and hybrid	1·2748
Total ÷ 5	0·5407

It is clear that the significant variance is between the two parents and between the hybrid and parent 1, i.e. the two parents had different relative growth rates, and the hybrid had a relative growth rate not significantly different from that of one parent.

3. *Dry weight data.* Experiment 2. Table XIV contains an analysis for dry weights in experiment 2. Strain variance is again significant; so also are variances due to time and block. There was no significant variance in the interaction of strain and time. The totals of logarithms for the three strains were:

No. of entries.	Parent 1.	Parent 2.	Hybrid.
48	113·076	120·825	123.225

The standard error is 0·137. From partition of this sum of squares it emerges that the significant difference lies between the two parents. The hybrid is not significantly heavier than parent 2. There is no significant size-heterosis in this experiment.

TABLE XIV

Variance due to	d.f.	Sums of sq.	Var.	'z'	1% point.
Strains	2	1·17229	*0·58614*	1·714	0·764
Blocks	3	0·36288	*0·12096*	0·949	0·665
Time	5	129·01057	*25·80211*	3·614	0·552
Strains × time	10	0·23359	0·02336	0·160	0·460
Blocks × time	15	0·37963	0·02531	0·150	0·391
Blocks × strains	6	0·03224	0·00537	—	—
Triple interaction	30	0·63015	0·02100	0·057	0·291
Error	72	1·34967	0·01875	—	—

4. *Dry weight data.* Experiment 3. The analysis of variance is set out in Table XV. Strain, time, block, and the interaction of strain and time are all significant. The totals of logarithms for the three strains are:

No. of entries.	Parent 1.	Parent 2.	Hybrid.
36	78·1667	72·5617	88·7575

The standard error is 0·0916. When the sum of squares is decomposed it is established that the parents differed significantly in weight, and the hybrid was significantly heavier than either of them. Heterosis is therefore demonstrated.

The interaction of strain and time indicates a significant difference in efficiency index between the three strains. The sums of squares in this interaction are distributed as follows:

Between parents 1 and 2 . . .	0·27123
Between parent 1 and hybrid . . .	0·09875
Between parent 2 and hybrid . . .	0·26881

It is clear from these figures that the efficiency index of the hybrid is not different from that of parent 1. The two parents differ significantly in efficiency index, and the hybrid inherits the higher rate.

TABLE XV

Variance due to	d.f.	Sums of sq.	Var.	'z'.	1% point.
Strains	2	3·7582	*1·8791*	2·7058	0·764
Blocks	2	0·0915	*0·0485*	0·8488	0·764
Time	3	111·7711	*37·2570*	4·1981	0·665
Strains × time . . .	6	0·4260	*0·0710*	1·0677	0·515
Blocks × time . . .	6	0·0379	0·0063	—	—
Blocks × strains . .	4	0·0854	0·0214	0·4681	0·599
Triple interaction . .	12	0·0311	0·0026	—	—
Error	72	0·6059	0·0084	—	—

5. *Height data.* Experiment 1. In Table XVI are set out the results of an analysis of variance of logarithms of plant height. Variance due to strain and time are significant. The totals of logarithms for the three strains are:

No. of entries.	Parent 1.	Parent 2.	Hybrid.
48	46·238	41·646	50·493

The standard error is 0·0646. It appears from partition of the sum of squares that parent 1 is significantly taller than parent 2, and the hybrid is taller than either parent. There is significant height-heterosis.

TABLE XVI

Variance due to	d.f.	Sums of sq.	Var.	'z'.	1% points.
Strains	2	0·81570	*0·40785*	2·2903	0·764
Blocks	3	0·01515	0·00505	0·0944	0·665
Time	5	25·07415	*5·01483*	2·3935	0·552
Time × strain . . .	10	0·09829	0·00983	0·4276	0·460
Blocks × time . . .	15	0·06405	0·00427	0·0104	0·320
Blocks × strains . . .	6	0·01542	0·00257	—	—
Triple interaction . .	30	0·07347	0·00245	—	—
Error	72	0·30079	0·00418	—	—

6. *Assimilation rate.* Experiments 1 and 2. In Table XVII is given an analysis of the mean assimilation rates, from which it is evident that there is no significant difference in assimilation rates among any of the strains in experiments 1 and 2, when their variance is compared with the remainder variance.

TABLE XVII

Assimilation Rates from Experiments 1 *and* 2, *taken together*

Variance due to	d.f.	Sums of sq.	Var.	'z'.	1% points.
Strains	5	0·13666	0·02733	0·332	0·758
Blocks	3	0·24920	0·08307	0·888	0·845
Remainder . . .	15	0·21099	0·01406	—	—

The block variance is significant compared with the remainder. This is attributable to inequalities of illumination in the greenhouse.

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Number and Behaviour of Chromosomes in Aloe litoralis

BY

T. K. KOSHY, M.A., PH.D. (LOND.)

(Professor of Botany, Trivandrum, Travancore)

With Plate I and one Figure in the Text

I. INTRODUCTION

THE structure of chromosomes and the time and mode of their division are problems which are engaging the serious attention of cytologists at the present time. It was assumed till recently that each chromosome was built up of a number of definite morphological units (chromomeres) arranged in a series along a thread. This view came to be seriously questioned in 1926, when Kaufmann recorded in the chromosomes of *Tradescantia pilosa* a pair of unbroken intertwined spiral threads. Such threads were observed in chromosomes by several earlier workers, beginning with Baranetzky (1880). Vejdovsky (1912) noted similar threads in the chromosomes of Ascaris and he gave the name 'chromonema' to the thread. These observations, however, did not gain general recognition, as few investigators before Kaufmann succeeded in tracing the chromonema in more than a few phases in the history of chromosomes. With recent improvements in cytological technique and the stimulus given by the work of Kaufmann (1926) and Sharp (1929), considerable progress has been made in our conception of the behaviour of chromonemata in cellular activities. We now believe that the chromatic part of each chromosome is composed of a pair of coiled chromonemata and not of homogeneous 'beads' of chromatin, the chromomeres, strung together.

The general acceptance of the chromonema theory has not, however, cleared up certain important aspects of chromosome behaviour. In the history of chromosomes there are two stable systems, the resting nucleus and the metaphase chromosomes. Duplication of each chromosome should occur in one of them, and having regard to the coiled nature of the chromonema, the mode of cleavage and separation of the coils are fresh problems which require careful study.

Four years ago the author undertook a detailed study of these problems under the guidance of Professor Ruggles Gates, at King's College, London, and the results of his investigations on the chromosomes of several species of Allium were embodied in two papers (Koshy 1933, 1934). The following conclusions were then arrived at:

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Mitosis

1. Each chromosome is composed of a pair of chromonemata which are twisted on each other in all phases of its history.
2. During prophase contraction a secondary spiral originates in each thread, so that a 'spiral-twisted-on-spiral' will be formed in each chromosome.
3. This spiralization reduces the number of twists between the component threads of each chromosome. The 'remnant' twists of these are removed at the metaphase by a process of unwinding prior to their separation.
4. The compactly coiled chromonema of each chromatid of the late prophase or early metaphase undergoes cleavage along the thread, so that each daughter chromosome is composed of a double interlocked coil.
5. By a process of expansion characteristic of the telophase the double interlocked coil of each chromosome gets drawn out into a twisted thread giving the so-called 'alveolar' and 'chromomeric' appearances in successive stages of this expansion.

Meiosis

The behaviour of chromonemata in meiosis is essentially similar to that in mitosis. The leptotene threads are double and twisted on each other. Spiralization of each thread during prophase gives rise to a pair of nearly independent spirals in each chromosome. But cleavage is suppressed; and consequently homologous chromosomes pair, developing the same quadruple structure at the metaphase of the heterotypic division as in somatic metaphase. The paired bivalents separate, each anaphase chromosome having a pair of coiled chromonemata.

In a comparative study of the somatic and meiotic chromosomes of *Scilla nonscripta*, Miss Hoare (1934) reached nearly identical conclusions. The sequence of events in the history of chromosomes of Allium has also in part been confirmed by more recent workers in both plant and animal chromosomes. But as some of these observations are new, an extensive study of chromosome behaviour in a wider range of material was undertaken on my return to Travancore, with a view to finding out the extent to which the above scheme was of general application. The present paper gives an account of the chromosomes of *Aloe litoralis*.

It is also proposed to discuss the mechanics of mitosis in the light of our present knowledge of the structure of chromosomes. In doing so, the author wishes to record his grateful appreciation of the keen interest which Sir John Farmer evinced in these problems and the continued help of Professor Ruggles Gates.

II. MATERIAL AND METHOD

Aloe litoralis Koenig is a liliaceous plant growing wild in the sandy tracts along the coast of Travancore, abounding chiefly at Cape Comorin. It has a prostrate stem with a rosette of thick fleshy leaves. The flowers are borne on an erect axis which shoots up from the centre of the rosette with the commencement of the monsoon rains in May. It is a plant of considerable medicinal value, the juice from the succulent leaves being used 'externally in cases of ophthalmia, and especially in what are commonly termed country sore-eyes' (Drury, 1858).

A preliminary examination of flower buds collected from Cape Comorin in September 1934 showed that the plant has only a limited number of chromosomes, some of which are sufficiently large for a critical examination of their internal structure. The cytology of this species does not seem to have been worked out, and its chromosome number has not yet been ascertained. An extensive collection of material was therefore made in the following year. Flower buds in all grades of development were fixed at the Cape in the following fluids: Flemming's medium fluid; Navashin's fluid; La Cour's fluids 2 B and 2 BD; and Benda's modification of Flemming's fluid. A previous treatment of each flower in Carnoy's mixture for a few seconds was given before transferring it to the fixing fluid. The flowers were collected between 9 a.m. and 1 p.m. and fixed for 4–6 hours. Smears were also made and fixed in Flemming's medium fluid and La Cour's 2 B fluid. Paraffin material was cut 4–12μ thick, and stained in gentian violet. In the description of plates particulars regarding fixing fluid employed and other details of the material relating to each figure are given.

III. NUMBER AND MORPHOLOGY OF CHROMOSOMES

Aloe litoralis Koenig has fourteen chromosomes in the diploid nuclei. These range themselves into three distinct types:

three pairs of large chromosomes (l_1, l_2, l_3),
two pairs of median (m_1, m_2), and
two pairs which are very small (s_1, s_2).

These chromosomes as seen in the early metaphase of a somatic set are shown seriatim in Pl. II, Fig. 1; and they are designated for convenience of description as l_1, l_2, l_3, m_1, m_2, s_1, s_2. The outstanding morphological features of these are given in the adjoining Table.

Chromosome.	Approximate length.	Position of constriction.	Length. Long arm.	Short arm.	Thickness.	Shape.
l_1	12 μ	Sub-terminal	10	2	2 μ	J
l_2	12 μ	Sub-median	8	4	1·5 μ	J
l_3	10 μ	Sub-terminal	8	2	2 μ	J
m_1	10 μ	Median	5	5	1 μ	V
m_2	9 μ	Median	5	4	1 μ	V
s_1	6 μ	Sub-terminal	4	2	1 μ	J
s_2	5 μ	Sub-terminal	4	1	1 μ	I

This grouping of the chromosomes of *Aloe litoralis* coincides in essential details with the chromosomes of *Aloe vera* L., worked out by Sutaria (1932).

In the latter species, however, the m_1 chromosome appears to be longer than in the former, and consequently in Sutaria's descriptions we see four pairs of long chromosomes and only three pairs of short chromosomes.

IV. BEHAVIOUR OF CHROMOSOMES

(a) *Somatic chromosomes*

As stated in the Introduction, this study was undertaken chiefly with a view to ascertaining the mechanics of mitosis in relation to the spiral structure of chromosomes. Particular attention was therefore given to all evidence relating to these problems without limiting the account to a detailed description of the mitotic phenomena of cells.

Metaphase. After the chromosomes have reached a certain stage of contraction the nucleolus and the nuclear membrane disappear. Pl. I, Fig. 2, is a polar view of the chromosomes at this stage. All the fourteen chromosomes of the complement are seen here, and they are shown by letters *l–s*, in Pl. I, Fig. 2 *a*. Each chromosome is distinctly double and their halves, while generally free in the short chromosomes and short arms of long chromosomes, are clearly seen twisted on each other in the long arm of chromosomes l_1, l_2, and l_3. Even in small chromosomes there are clear indications of their halves having been twisted on each other, as may be seen by the persistence of half-twists in almost all of them. In the long chromosomes, the long arm of each shows about three full twists and one (rarely two) full twist in the short arm. It was pointed out in an earlier paper (Koshy, 1933) that the twisted halves of each chromosome get separated by a process of unwinding which commences at the ends of each chromosome. In the long arm of l_1 chromosome of Pl. I, Fig. 2, this is seen clearly. There is also a reversal in the direction of twisting of the two halves with reference to the attachment constriction (kinetochore) of each chromosome. In all cases where the direction of twisting could be traced out, this feature appeared to be a constant character in chromosomes. Although this twisted aspect of early metaphase chromosomes is seen figured in several cytological papers, its significance appears to have escaped serious notice. Pl. I, Fig. 3, shows the metaphase chromosomes after they have arranged themselves at the centre of the cell. Each chromosome now gets fixed to the plate by means of fibres extending polewards. The kinetochores are in the same plane and the chromosome arms extend in all directions from the plate. The twisted halves gradually separate by unwinding. A remarkable feature of the chromosomes at this stage is the moniliform appearance presented by them in well-fixed preparations. A comparison of chromosomes of Pl. I, Figs. 2 and 3, would show how the latter assume a wavy margin as metaphase advances. The cut ends of these present the appearance of hollow cylinders. With the organization of the nuclear spindle and the removal of twists between chromosome halves, the daughter chromosomes are ready to get pulled apart.

Anaphase. The short chromosomes of the complement move apart and reach the poles earlier than the long ones. The movement of the latter is a slow process, all grades of their separation being easily noticed in the different cells of the same section. A detailed examination of these chromosomes in both deep- and faintly-stained preparations has afforded the clearest indications of their dual nature. In several cases forked ends were distinct, and the twisted nature of the component strands was seen clearly in a few cases (Pl. I, Fig. 4). These are shown by arrow marks in the figure. These, with the moniliform appearance they present, should leave no doubt as to their dual nature.

As the chromosomes reach the poles their chromonematic coils come closer together, forming the so-called 'tassement polaire' stage. This stage, however, is of short duration in Aloe as the coils soon expand in preparation for the resting stage.

Telophase. An outstanding feature of the chromosomes at this stage is their tendency for expansion. As the stage advances the chromosomes become thinner and clear diamond areas appear along the coil of each. The origin of such areas has been recorded by several investigators beginning with Grégoire and Wygaerts (1903), and they were interpreted as vacuoles (alveoles) arising *de novo* in the homogeneous matrix of chromosomes. This conclusion has already been shown to be unsound as these areas are the direct result of the expansion of interlocked double coils of each chromosome. Pl. I, Fig. 5, shows a telophase nucleus in which such areas are seen clearly. A nuclear membrane is now organized round each daughter nucleus, restricting thereby the straight expansion of the coils of each chromosome. These are consequently thrown into zigzag folds. The diamond areas diminish in size and the dual chromonemata are drawn out into a twisted thread.

Resting stage. These twisted threads thrown out into zigzag structures naturally form a tangle of threads at the resting stage. They are so thin that neither their dual nature nor twisted aspect can be made out in the interphase nuclei. It may be pointed out that the extension of the chromonematic coil which leads to the organization of the resting nucleus does not affect the length of the threads or their individuality. If a thin wire 0·5 mm. thick is wound round a rod of diameter 2 mm., about 12·5 cm. of wire would be necessary to make a compact coil of 1 cm. with 20 gyres. If two such wires are held parallel and wound round the rod, a double interlocked coil (2 cm. long) would be formed. Such a double coil, if pulled from the ends, would first form diamond areas along the coil and finally give rise to a twisted thread. The number of twists would be equal to the number of coils and the maximum expansion about twelve times the length of the coil. In the passage of anaphase chromosomes into the resting stage a similar expansion of their chromonematic coils is assumed to occur; consequently the coiled chromonemata are relaxed into twisted threads. These being so delicate and thin, hardly any details of their internal structure or organization would come within the range of visibility. In the earlier stages, however, the chromonemata being in a coiled state, compact 'hollow cylinders' are formed by them. Individual chromosomes are thus easily seen in these stages.

Prophase. In the actively dividing regions of the plant body the resting stage is of short duration. With the onset of mitotic activity, the twisted chromonemata are seen to assume peripheral positions in the nucleus, forming a hollow sphere (Pl. I, Fig. 6). The twisted threads then begin to contract. Gradually shorter and thicker chromosomes are formed in each nucleus (Pl. I, Fig. 7). Although the zigzag folds of each chromosome persist, distinctly twisted structures are presented by chromosomes at this stage. In Pl. I, Fig. 6, which is from a less-stained preparation, the interlaced aspect of the component threads may be seen very clearly. Pl. I, Fig. 7, which is from a deeply stained slide, shows its chromosome outlines more markedly with less conspicuous diamond areas.

Special attention may here be drawn to two outstanding features of prophase chromosomes, viz. their mode of contraction and twisted nature. There is no doubt that as prophase advances shorter and thicker chromosomes are organized in each nucleus. During contraction each thread of the twisted pair spiralizes independently, giving rise to a spiral-twisted-on-spiral structure. The phenomenon of coiling is perhaps associated with the conservation of space, as Kaufmann (1926) has suggested, and it is the natural result of the arrangement of the particles composing the thread in a spiral manner. This mode of contraction (Kuwada, 1927) gives rise to compactly coiled chromonemata in each chromatid.

At the late prophase the nuclear membrane disappears. Cleavage occurs along the coiled thread of each half and the chromosomes arrange themselves at the equator of the spindle. There they get fixed by spindle fibre attachments and their halves unwind prior to separation.

(b) *Meiotic chromosomes*

The behaviour of chromonemata in meiosis is essentially similar to that in mitosis. The significant facts connected with this phenomenon are the pairing of homologous chromosomes, and the reduction of the diploid ($2n$) number of chromosomes of the mother-cell to the haploid (n) number. Although these are accomplished during the first (heterotypic) of the two divisions of meiosis, why an apparently normal (homotypic) division should immediately follow the heterotypic division is not yet clearly understood. The chief point on which discussion has centred in recent years is whether the early prophase threads (leptonema) in preparation for meiotic divisions are single or dual structures. Those who advocate interphase cleavage in mitosis (Darlington and others) advance the view that cleavage is suppressed at the early prophase stage of meiosis so that the threads emerge from the resting nucleus as single. Huskins and Smith (1934) are of opinion that cleavage is partly or wholly suppressed at the metaphase of the last premeiotic division and consequently, at the time of pairing, each strand is composed of a single thread. As opposed to this, the author (1934) has advanced the view that the early leptotene threads are composed of double, twisted chromonemata. Hoare (1934) came to the same conclusion from her work on *Scilla*. Gates and Nandi (1935) have observed in a wild species of Oenothera the leptotene threads as split like the threads in somatic prophase with the halves twisted about each other. *Aloe litoralis* has afforded further evidence in support of this view.

Leptonema stage. At the earliest prophase of meiotic division the leptonema presents a corrugated appearance (Pl. I, Fig. 8). Imperfect fixation of these delicate threads may to some extent result in the retention of interchromosomal strands of karyolymph giving the nuclear contents a reticulate appearance. The threads may also present a 'beaded' aspect. But careful examination of these in faintly stained regions of the cell has shown that each strand is composed of two chromonemata twisted about each other. With the rapid increase in size of the nucleus, the threads assume peripheral positions in the nucleus, forming a hollow sphere enclosing one or more nucleoli. Most of the folds and bends of the threads are lost and they are seen fully stretched out on the surface of the hollow sphere. Contraction of the threads commences immediately after the threads have thus arranged themselves in the nuclear chamber.

Zygonema stage. The threads at this stage also exhibit a tendency to associate in pairs. As recorded in Allium, the pairing was seen here also to commence at the ends of the homologues (Pl. I, Fig. 9), and proceeding gradually towards their kinetochores. The chromonemata now present a crenulate margin, and clear cases of strands crossing each other were observed in a few bivalents. Pl. I, Fig. 10, represents a stage when pairing is complete. The mates remain side by side and in close association with each other. As contraction is proceeding simultaneously the pairs are soon organized into short thick bivalents (Pl. I, Fig. 10). They also exhibit a marked affinity for stains. It has been difficult, therefore, to follow up carefully their internal structure. However, in favourable portions along the chromosomes an undulating contour and parallel rows of granules were clearly seen. These should certainly be indications of the organization of a pair of *free* spirals in each homologue. It was assumed (Koshy, 1934) that the interlaced chromonemata of each chromosome get resolved into two independent coils at the zygonema stage by a reversed twist occurring simultaneously with spiralization. This spiralization should satisfactorily explain the resolution of the twisted components into two free coils. It has been shown above that about 12·5 cm. of wire of 0·5 mm. thickness would be necessary to make 20 coils of diameter 2 mm. If these coils are compressed it would be about 1 cm. long. A similar double coil made of two wires of the same length and thickness if pulled from the ends would form a cord about 12 cm. long with the halves twisted about one another twenty times. Now, if each wire of the cord independently forms a coil of the same diameter (2 mm.) the length of the coil would be only one-twelfth of the length of the thread. This contraction by spiralization may thus be powerful enough in chromosomes to remove nearly all twists between its component threads. A pair of free coils is thus organized in each prophase chromosome. The synaptic association of homologous chromosomes occurs concurrently.

Diplonema stage. The side-by-side association of the pairing chromosomes is soon disturbed by the appearance of loops between the pairs (Pl. I, Fig. 10). These loops originate at the constriction region of each bivalent. This may probably be due to the repulsion between chromosomes which manifests itself soon after pairing. This repulsion soon proceeds towards the ends, and consequently the loops become wider and the homologues gradually lose contact with each other except at their ends. Ring bivalents are thus formed. In studying the configurations of the bivalents of Aloe from diplotene to metaphase, this mode of ring formation was seen in all the seven pairs of the complement. The first ring to appear is the shortest chromosome pair, s_2. This is soon followed by the s_1 pair. In Pl. I, Fig. 10, these two rings are seen. At a slightly later stage the rings of m_1 and m_2 appear. Meanwhile, the ends of the short arms of s_1 and s_2 pair separate, forming open rings. Finally, at the fully contracted state of the bivalents (diakinesis) the three rings of the large chromosome pairs (l_1, l_2, l_3) are invariably met with (Pl. I, Fig. 11). The bivalents in such varying grades of open and closed rings arrange themselves at the metaphase plate (Pl. I, Fig. 13). Thus, side-by-side association and loop formation account for the different diakinetic figures presented by the bivalents of *Aloe litoralis*.

While only a single loop is seen in small chromosomes, the large chromosomes show at this stage two or even three loops. By a twist in the ring, figures of eight are usually formed in long chromosome pairs (Pl. I, Fig. 12). Clear cases of strands crossing over at a few points along the bivalents were observed in this material (Pl. I, Fig. 9). Whether actual interchange of partners occurs at these points, or whether they are only due to the twisting of these as a result of the differential contraction of chromosomes, could not yet be ascertained.

Metaphase. Pl. I, Fig. 14, shows the forms generally presented by the bivalents at the heterotypic spindle. At this stage all of them appear as open rings with only one end of the homologue in contact with the corresponding end of its mate. The dual strands of each are clearly seen at this stage. Although these strands have a corrugated structure, being thrown into folds during the preceding prophase contraction, they do not show any indication of intertwining. In Allium a few twists were seen to persist in metaphase bivalents. Such twists are absent in Aloe. No prior unwinding is therefore necessary for the separation of the univalents.

Anaphase. As in Allium the smallest pair effects the separation of its halves earlier than the rest (Pl. I, Fig. 13). As the chromosomes move to the poles the two compactly coiled strands of each also move apart retaining contact only at the attachment constriction. Characteristic X-shapes (Pl. I, Fig. 15) are thus presented by anaphase chromosomes. In this important character these chromosomes differ from similar chromosomes of somatic division where the two chromonemata of each chromosome exist as an interlocked double coil, whereas two independent coils are present in each chromosome at this stage.

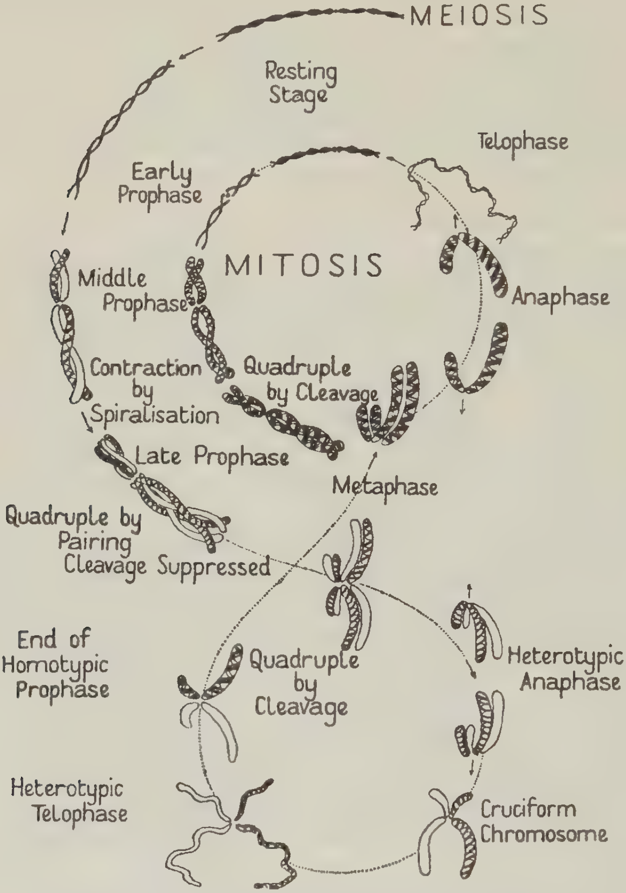
Telophase. The quadripartite chromosomes which converge towards the poles gradually extend their arms, exhibiting the same tendency for expansion of the coils. As each diverging arm is only formed of a single-strand coil, the diamond areas which are formed during such expansion in normal telophase are significantly absent. The chromosomes pass on rapidly to the second maturation division without an intermediate period of rest.

Second maturation division

The quadripartite figures of the previous anaphase and telophase are still clearly made out in the early stages of the homotype division (Pl. I, Fig. 16). But the polarization of the chromosomes is lost and they are arranged irregularly in the nucleus. Their diverging arms do not show any indication of a twisted aspect. At the homotypic spindle they arrange themselves in such a way that their arms are disposed symmetrically with reference to the plate (Pl. I, Fig. 17). The chromosomes are longer and thinner, appearing as straight rods. Their halves are in contact only at their kinetochores, and consequently their separation does not involve any prior untwisting. Anaphasic separation commences at the constriction and the J-shaped chromosomes gradually move apart. Clearest indication of the dual nature of each chromosome was seen at this stage (Pl. I, Fig. 18). They show not only a moniliform appearance but also (in faintly stained preparations) the dual, interlaced aspect. The homotypic anaphase chromosomes thus present an identical structure to somatic anaphase chromosomes (cf. Pl. I, Fig. 4). Pl. I, Fig. 19, is a polar view of a haploid complement at the anaphase.

The tetrads are soon organized, and in the constitution of each nucleus the diamond areas formed by the expanding double interlocked coil of each chromosome were clearly observed at the homotypic telophase (Pl. I, Fig. 20). The meiotic events in the microsporogenesis of *Aloe litoralis* are thus completed.

These details of structure and behaviour in both somatic and meiotic chromosomes are shown in the Text-figure p. 52. The two chromosomes presented for comparison follow the same sequence of events in the respective division of each and have nearly identical structure. While a quadruple structure is developed in each chromosome at the late prophase of mitosis by cleavage in each chromonema, the pairing of homologous dual chromosomes and consequent suppression of cleavage at the heterotypic division establishes a similar condition in meiosis prior to the separation of the paired halves of each quadruple bivalent. The only difference between the two types is that while each anaphase chromosome of normal mitosis has a two-strand coil, the heterotypic anaphase chromosome has two one-strand coils. As the former is the more stable condition in chromosomes, a second (homotypic) division is rapidly undertaken in order that this balance may be re-established by cleavage in the chromonema of each coil. The homotypic anaphase chromosome has thus the same two-strand coil as in somatic chromosomes.



Behaviour of chromosomes in mitosis and meiosis.

V. Discussion

(a) *Spiral structure of chromosomes*

The structure of large chromosomes in both plant and animal material has been thoroughly examined in recent years by a large number of investigators, and there is general agreement as regards plants that the chromatic part of each chromosome exists as a delicate thread, the chromonema, which maintains a coiled aspect in all stages of its history; although there is difference of opinion as to the number of threads which constitute a chromosome coil. Kuwada and his co-workers in Japan support the view that each chromosome during 'pre-resting' stages is composed only of a single coiled chromonema. They assume that this coil is drawn out into a straight thread prior to longitudinal split at the resting stage so that two free parallel threads become clearly marked out in each chromosome only from the earliest prophase. The two halves then spiralize independently, forming a pair of free coils which separate at the following anaphase. Darlington (1935) is also in favour of interphase cleavage in chromosomes; but he thinks that the coiled aspect is retained in the chromonema at the resting stage and that by a 'helical' plane of cleavage two free coils are formed in each chromosome. These investigators thus uphold the view that pre-resting chromosomes are unitary structures, duality commencing in each only at the interphase.

The number of threads which are actually present in a chromosome coil may be determined both by observational evidence and by inference. It was reported by many investigators (Kaufmann, 1926; Sharp, 1929; Telezynski, 1930; and Koshy, 1933) that in somatic mitosis the two chromonemata of each chromosome are twisted on each other. Kaufmann's (1934) figures of anaphase chromosomes in the somatic cells of *Drosophila melanogaster* show unmistakable evidence of such split, twisted chromosomes. Kuwada and Nakamura (1935) have observed in Fritillaria and in living staminate hair-cells of Tradescantia the two chromonemata of each chromosome twisted on each other in the telophase. In *Aloe litoralis* the twisted aspect of chromosomes was clearly seen not only in the anaphase and telophase stages but also in the prophase and early metaphase. How can we account for the persistence of this twisted aspect in chromosomes?

It was pointed out in my previous paper on Allium that the diamond areas which were seen in telophase and prophase chromosomes by earlier workers and interpreted by them as alveoli are only areas formed during the expansion of an interlocked double coil. There is no doubt that such clear areas are invariably met with in telophase and prophase chromosomes. Now, if as assumed by Kuwada, Darlington, and others, the pre-resting chromonematic coil is formed of a single thread, how can we get those twisted appearances in chromosomes? Again, what explanation can be offered for the occurrence of alveolar areas characteristic of telophase chromosomes? A coil formed of a single thread cannot by its expansion give rise to such areas. There is no doubt, therefore, that two coiled chromonemata are present in each chromosome in all stages of its history.

It was also pointed out by the author that Earl's (1927) view of the gene thread as the basis of chromonema was strictly in accord with the above observations and that it was highly probable that the particles which compose the thread were arranged *spirally* along its axis. Darlington (1935) has advanced this as a view of his own and has used the term 'molecular spiral' for the coiled thread of such organization. Each chromonema is thus assumed to be composed of spirally arranged particles (genes). It originates as a spiral and persists as such in all phases of its developmental history.

(b) *Time and mode of cleavage*

The stage at which cleavage occurs in mitosis is still a debated problem. As shown above, Kuwada, Darlington, and a few others still persist in the view that cleavage is an interphase phenomenon. As opposed to this, Hedayetullah (1931) working on Narcissus, and Perry (1932) on Galanthus, advanced the view that cleavage is initiated in chromosomes as early as the metaphase of the previous division. They observed quadruple strands in metaphase chromosomes and dual chromonemata in anaphase and subsequent stages. This observation has been confirmed in Allium (Koshy, 1933) and Scilla (Hoare, 1934) as well as in the present material, *Aloe litoralis*. Most of the recent workers are also in favour of the latter view. It is fairly certain, therefore, that cleavage originates in chromosomes at or just before metaphase of the preceding somatic cycle. There is, however, considerable divergence of opinion regarding the mode of chromosome cleavage. The acceptance of a spiral structure in chromosomes has naturally upset our current ideas on the mechanics of mitosis. If cleavage occurs when the chromonema is in a coiled state, what is the line of cleavage, and how do the halves separate?

Kuwada and Nakamura (1935) assume that at the time of cleavage the chromonemata are drawn out into straight threads, a pair of parallel threads developing from each by longitudinal cleavage. These, according to them, spiralize independently forming a pair of one-strand coils in each chromosome. Darlington (1935), on the other hand, thinks that by a 'helical' plane of cleavage in each coiled chromonema, a pair of free coils are formed in each chromosome. This mode of cleavage is similar to that suggested by Nebel (1932). Nebel has claimed that two free coils, one fitting into the other, will be formed if the duplication of the coiled thread 'occurs in a plane parallel to one (and only one) plane passing through the main axis of the chromonema'. This conclusion has been shown to be geometrically unsound, as only two *interlocked* (and not free) coils would be formed by this mode of cleavage. If cleavage occurs when the parent chromonema is in a coiled state, the plane of cleavage has naturally to rotate with reference to the axis of the coil. The resultant halves consequently cannot but be interlocked. The views of Hedayetullah and Perry (loc. cit.) on this problem are also not in accordance with the accepted spiral structure of chromosomes. They assume that the chromonema is a straight thread (and not a coil) when cleavage occurs. Two parallel strands originate from each by longitudinal split. If, as we believe, the chromonemata are coiled into compact spirals at the metaphase where cleavage occurs, a straight split would cut across the spiral. And further, this mode of origin of the dual strands would not satisfactorily explain either the twisted aspect of anaphase chromosomes or their alveolar or chromomeric appearance in subsequent stages.

The last view on this problem is that suggested by the author. It is assumed that cleavage which occurs at the metaphase of the previous division is in a spiral direction with reference to the axis of the coil. By this mode of cleavage which may be termed 'spiral cleavage', a two-strand interlocked coil is formed in each chromatid prior to the separation of sister chromatids at the somatic metaphase. In the passage of each daughter chromosome to the resting stage, its two-strand coil expands, giving rise to twisted, alveolar, and chromomeric appearances in successive stages. It is fully drawn out into a twisted thread at the resting stage. With the onset of mitotic activity the chromosomes show extreme contraction. This may happen in one of three ways. The two threads of each chromosome may undergo linear contraction forming shorter and thicker chromosomes; or by the contraction of the matrix surrounding the threads, the twisted chromonemata may be compressed into zigzag structures; or finally, each thread may spiralize independently, forming secondary coils in each chromosome. The first of these processes would destroy the individuality of the chromonema, the second would upset its spiral structure, while the third would maintain both. Although it is difficult to advance evidence in support of any one of these, the sequence of events in the history of chromosomes and the accepted genetical role of chromonemata seem to favour the third view. As a result of this mode of contraction, a secondary spiral is developed in each chromonema. Consequently a spiral-twisted-on-spiral structure is assumed by chromosomes towards the end of prophase. Each chromosome has thus two one-strand coils twisted on each other. As already explained, the major twists of these are removed by unwinding at the metaphase. Cleavage for the following mitosis also occurs simultaneously.

Kuwada and Nakamura (1935) have reported doubly coiled chromonemata in the anaphase chromosomes of *Tradescantia reflexa*. In the somatic chromosomes of *Aloe litoralis* no clear evidence of such (doubly coiled) chromonemata was available at any stage before interphase, although indications of major and minor spirals were abundant in its heterotypic chromosomes. It is not unlikely that secondary spiralization, which ordinarily manifests itself in the prophase, may commence in each thread soon after its organization by cleavage at the metaphase of the previous division. In short chromosomes this may even result in resolving the interlocked coil of each chromosome into two parallel coils as early as the anaphase. This is the kind of difference which might very well occur between different genera.

The general acceptance of a pair of coiled chromonemata in all phases of chromosome history would inevitably upset most of Darlington's hypotheses on the meiotic phenomena of cells. In his three articles on 'The Internal Mechanics of Chromosomes', published in the 'Proceedings' of the Royal Society (1935), he has endeavoured to explain the spiral mechanism of chromonemata in order that it might fit in with his hypotheses. It is unfortunate, however, that by his unsound reasoning and inaccurate conclusions he has only complicated the problem of the mechanics of mitosis. Several of Darlington's general assumptions regarding meiosis are based on the chromomeric view of chromosome structure. With the break-down of this view, his assumptions would also have to fall to the ground.

(c) *Behaviour of chromonemata in meiosis*

The most significant features of meiosis are the pairing of homologous chromosomes, their reduction from the diploid to the haploid number, and the close succession of a normal mitotic division to complete the meiotic processes of each mother-cell. Notwithstanding the divergence of opinion regarding the significance of the different processes involved in meiosis, a careful examination of the behaviour of the meiotic chromosomes of *Aloe litoralis* has confirmed the view of the author that meiosis is merely an elaborated mitotic division.

The early prophase chromosomes in preparation for the meiotic activity show the same dual interlaced structure. As in somatic prophase, spiralization occurs in each thread developing nearly independent coils in each chromosome. But cleavage is suppressed. Homologous chromosomes pair, and consequently the same quadruple structures (bivalents) are organized at the heterotypic metaphase. The paired halves then separate. The dual strands of each chromosome are now resolved into independent single-strand coils. In this important respect these chromosomes differ from those of normal somatic divisions. And, in my opinion, this feature determines the close succession of a mitotic (homotypic) division in each dyad in order that the balance between the component threads of each chromosome may be re-established. The two free coils of each chromosome pass on rapidly to the prophase of the homotypic division, without an intermediate period of rest. Cleavage is soon initiated, developing the same dual, interlocked coil. It is not unlikely, therefore, that a normal mitotic division closely follows the heterotypic in order to restore equilibrium between the component strands of each chromosome.

VI. SUMMARY

1. *Aloe litoralis* has fourteen chromosomes in the diploid nuclei.

2. Both somatic and meiotic chromosomes are composed of two-strand coils in all phases of their history.

3. In mitosis, cleavage is initiated in each chromonema before the separation of the daughter chromosomes.

4. The metaphase chromosome has thus a quadruple structure.

5. In meiosis, such quadruple structures are formed by the association of homologous pairs and there is complete suppression of cleavage in the first meiotic division.

6. While each anaphase chromosome of mitosis is composed of a two-strand coil, the anaphase chromosome of meiosis has its two strands resolved into independent coils.

7. As the former is the more stable structure in which the dual chromonemata associate in chromosome organization, a second (homotypic) division is rapidly undertaken in meiosis, which re-establishes by cleavage this balance between chromonemata.

8. Thus, in structure and behaviour the meiotic chromosomes are essentially similar to the somatic chromosomes.

In conclusion, the writer wishes to record his deep gratitude to Professor Ruggles Gates, for his continued interest in the progress of these investigations and for his helpful criticism in the preparation of this paper.

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EXPLANATION OF PLATE I

Illustrating Professor Koshy's paper on 'Number and Behaviour of Chromosomes in *Aloe litoralis*'

All figures (except Figs. 1 and 13) are drawn at table level with the aid of a Reichert camera lucida using a Leitz 1/12 oil immersion objective, aplanatic condenser, and Leitz ocular × 15. All figures (except Fig. 12) have a magnification of × 2,400. The fixing fluid used in the preparation from which the drawing is made, is Benda's modification of Flemming's fluid unless otherwise noted.

Fig. 1. The seven pairs of chromosomes of a diploid complement of *Aloe litoralis* arranged seriatim with reference to their size. The moniliform appearance of these chromosomes is indicative of the twisted aspect of the component halves at the early metaphase.

Fig. 2. Polar view of a metaphase plate. The seven pairs may easily be distinguished in the figure. These chromosomes are indicated by letters *l–s* in Fig. *2a*.

Fig. 3. Metaphase chromosomes arranged on the equator of the spindle. The two halves of each chromosome are twisted about each other and they show an undulating contour. The cut-ends of chromosomes present the appearance of hollow cylinders.

Fig. 4. Somatic anaphase. The chromosomes show a moniliform appearance and forked ends are noticeable in a few chromosomes. The twisted dual threads may be seen near the constriction of two chromosomes indicated by arrow-marks.

Fig. 5. A telophase nucleus. The diamond areas formed by the expanding chromosomes are seen more clearly near the attachment constriction of each.

Fig. 6. Early prophase. The two chromonemata of each chromosome are distinctly twisted about each other and show diamond areas between them.

Fig. 7. Late prophase from a deeply stained preparation. The chromosomes are stouter and shorter and the diamond areas less conspicuous. However, the twisted aspect of the component strands may be made out here.

Fig. 8. Early prophase of meiotic division. The leptotene strands present a granular appearance; but the dual interlaced threads which constitute each strand can be noted on careful examination.

Fig. 9. The zygonema stage. Pairing commences at the ends of the homologues and indications of the strands crossing between them may be seen. All the seven bivalents are shown. Flemming's medium fluid.

Fig. 10. Late prophase. The paired strands during contraction give a crenulate margin, and characteristic loops are apparent in each. The s_1 and s_2 pairs have become ring-bivalents.

Fig. 11. Diakinesis. The seven bivalents are seen in the figure. The s_1 and s_2 pairs are now only attached at one end, while the bivalents of l_1, l_2, and l_3 are nearly closed rings with figures of eight being formed by a twist in the ring of each.

Fig. 12. A magnified drawing of l_1 pair showing its internal structure and appearing as a figure of eight. Biseriate granules are seen along the chromosomes. This is indicative of the resolution of the twisted strands into free coils. × 3,400.

Fig. 13. Heterotypic metaphase, side-view, in which the seven pairs of bivalents are shown arranged at the plate. The shortest chromosome pair has just moved apart and the other bivalents are in all grades of open and closed rings.

Fig. 14. Similar to Fig. 13, being a slightly later stage. Most of the bivalents are in open rings with only one end of the homologue in contact with the corresponding end of its mate.

Fig. 15. An anaphase complement. The X-shapes and single-strand coil for each diverging arm are characteristic of these chromosomes. The halves are attached only at their kinetochores.

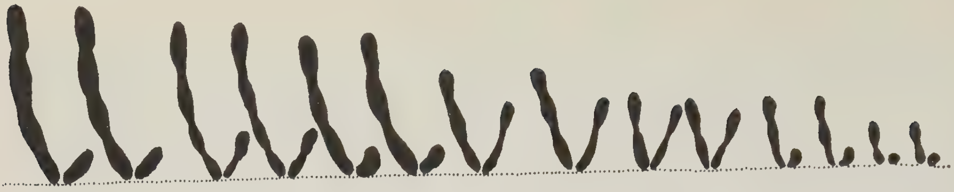
Fig. 16. Prophase of homotypic division showing the quadripartite figures with diverging arms. La Cour's 2 B.

Fig. 17. Homotypic metaphase. The chromosomes are disposed symmetrically with reference to the plate and no prior unwinding is necessary for the separation of their halves, as they retain contact only at their attachment constrictions.

Fig. 18. Anaphase of second division. Each chromosome presents a moniliform appearance. The dual interlaced aspect of each is clearly seen in some chromosomes.

Fig. 19. A polar view of the haploid chromosome complement towards the end of meiotic divisions from a deeply stained preparation fixed in Flemming's medium fluid.

Fig. 20. Telophase of second division. The diamond areas formed by expanding double coils may be seen in it.



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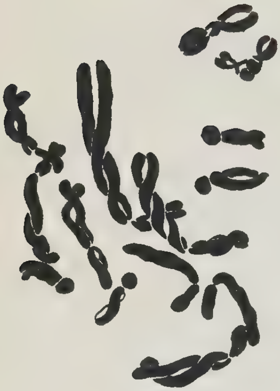

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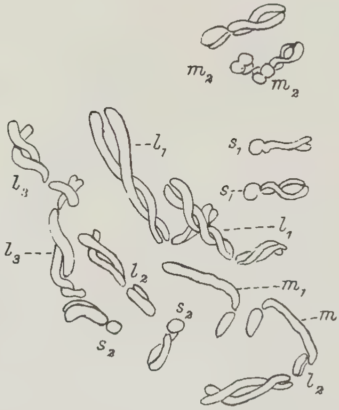

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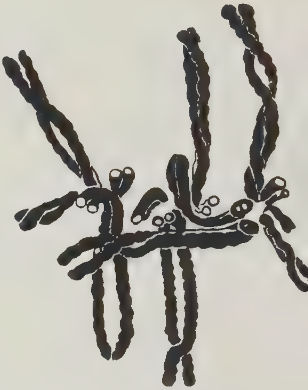

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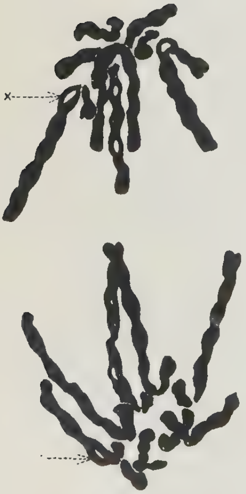

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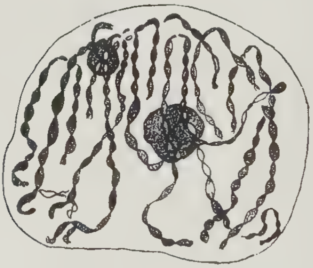

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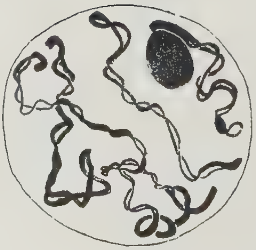

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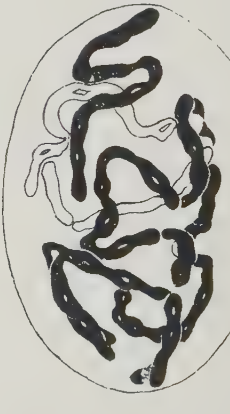

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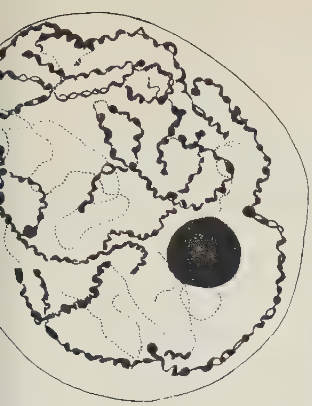
KOSHY – CHROMOSOMES OF ALOE LITORALIS.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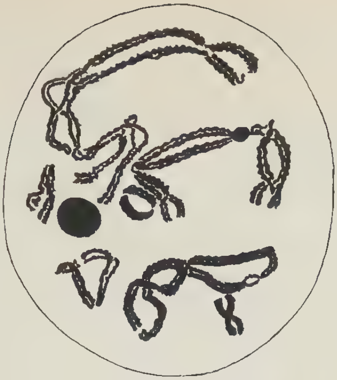
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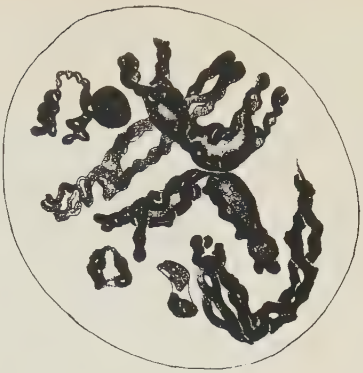
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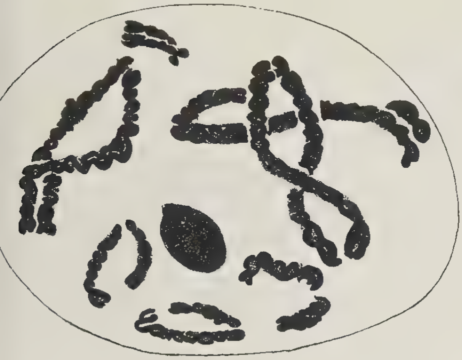
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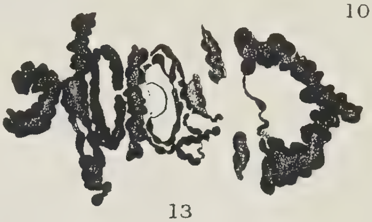
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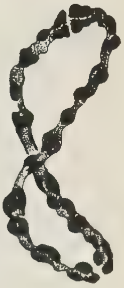
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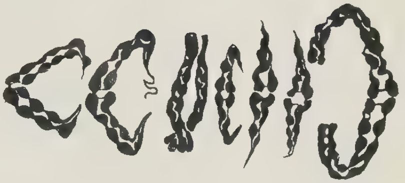
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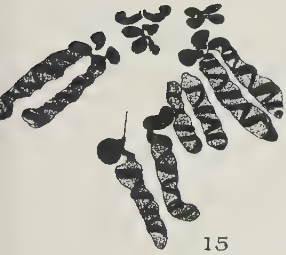
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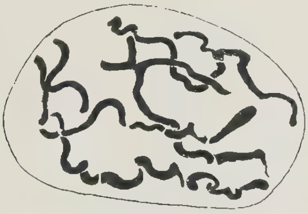
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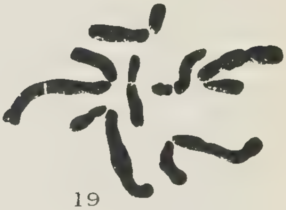
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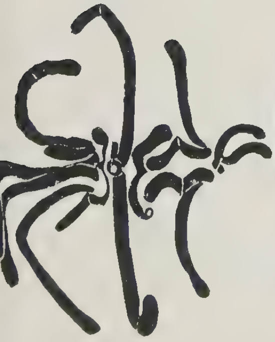
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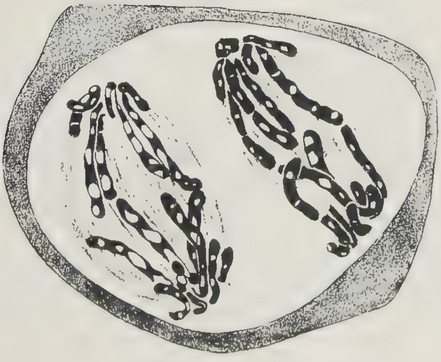
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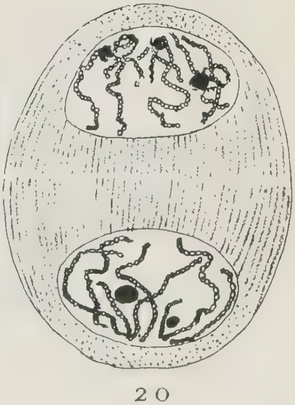
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Huth, Stubbs X., Kent

Studies in the Pathogenicity of Tropical Fungi

I. On the Types of Infection Encountered in the Storage of Certain Fruits.

BY

R. E. D. BAKER

(*Department of Mycology, Imperial College of Tropical Agriculture*)

AND

C. W. WARDLAW

(*Low Temperature Research Station, Imperial College of Tropical Agriculture, Trinidad, B.W.I.*)

I. INTRODUCTION: THE OCCURRENCE OF DORMANT OR LATENT INFECTIONS

IN considering the measures to be adopted in controlling wastage in stored fruit, a knowledge of the mode of infection of the several organisms involved and of their capacity for invading tissues at any given stage of maturity is essential. Even with some of the more common tropical pathogens, such information is still relatively scanty and incomplete.

While the extent to which different organisms can invade tissues may readily be ascertained in inoculation experiments, the actual liability of fruit to decay in storage is complicated by various factors. Among others the time and mode of infection are of considerable importance. The evidence available indicates that while certain kinds of rotting may be attributed to the activity of fungal spores alighting on the fruit after gathering, in other words to typical packing-shed organisms, many infections, among tropical fruits at least, are due to organisms which have become established as latent infections prior to picking. In addition to these, consideration must be given to the varied assemblage of spores which accumulate on the fruit surface during development. The distinction between superficially placed spores or conidia which have still to function, and those which have already germinated and effected an entry into the epidermal or sub-epidermal tissues of the fruit (i.e. latent infections) is, in the writers' opinion, of fundamental importance in determining the type of control measures to be adopted. Where the fungus is still superficial, fungicidal treatments applied with the object of killing the spores or germ-tubes may prove effective; but where the fungus has already penetrated the tissues (to remain as a dormant or latent infection until a certain stage of maturity has been reached) it is unlikely that such treatments will succeed.

Using tropical fruits, whose behaviour during storage is already sufficiently

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known, an attempt has been made, as a preliminary stage in these studies, to distinguish between those organisms which normally become established as latent infections and others whose pathogenicity only begins after fruits have been harvested. The factors which determine to which type a particular fungus belongs include the conditions affecting spore germination and the ability of the germ-tubes to penetrate the uninjured cuticle. Where latent infections are involved the fungus becomes established within the epidermis while the fruit is still immature, but further pathogenic activity is frequently, though not invariably,[1] arrested, the fungus remaining dormant until the fruit becomes more or less senescent with concomitant loss of resistance (Wardlaw and Leonard, 1935). At the time of picking, these infections are invisible or inconspicuous.

The activity of superficially placed spores (other than those which give rise to latent infections) is dependent on a number of factors for effective parasitism. These include the presence of sufficient moisture in the form of liquid or vapour to permit of spore germination and the occurrence of wounds by which access to the tissue is rendered possible. Not infrequently the development of the latent infections referred to above affords the necessary means of ingress. Once penetration is effected the secondary organisms may exploit the tissue more rapidly than the primary pathogens.

Hitherto, knowledge of latent infections as they occur under West Indian conditions has been deduced from the manner in which certain diseases develop in stored fruit rather than from experimental isolation. In the present paper an attempt has been made to establish, by mycological examination, the actual state in which the several potential pathogens are present. Particular attention has been directed to the grapefruit, but cognate observations on papaws, bananas, and mangoes are also submitted.

II. Previous Investigations

The presence of latent infections in the tissues of plants and fruits has been demonstrated from time to time by various investigators. The following technique was commonly used in such work. Leaves or fruits were surface-sterilized, generally with mercuric chloride, stored under sterile conditions, and the subsequent development of fungi, which were assumed to have been present in the tissues as latent infections, was noted.

Using this technique Shear and Wood (1913) showed that species of Glomerella and Colletotrichum were present in the tissues of many plants and fruits. Similarly, Dastur (1916) showed that *Gloeosporium musarum*, the cause of a fruit rot of the plantain in India, attacked the fruits long before they were ripe and remained dormant in the skin until suitable conditions arose for its development, and Hawkins (1921) found *Colletotrichum gloeo-*

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[1] An exception must be made of certain Phytophthora species which can penetrate the cuticle of immature citrus fruits and cacao pods and commence active invasion of tissues at once.

sporioides in the skin of grapefruit in storage. More recently Wardlaw and Leonard (1936) have demonstrated that *Colletotrichum gloeosporioides* attacks the mango in a similar fashion, and Wormald (1934) has concluded that many of the lesions of Apple Scab (*Venturia inaequalis*) which develop in stored fruit are initiated in the field. Kuntz (1932) and Tisdale and West (1934), working with citrus fruit in Florida, have isolated *Diplodia natalensis* and *Phomopsis citri* from surface-sterilized buttons of tree-ripe oranges and grapefruit. Recently, working on citrus, Bates (1936) has discussed latent infections and gives a bibliography.

TABLE I

Occurrence of Fungi in Surface Washings of Grapefruit

	Grapefruit surface washings.	
Organism.	Number of times isolated from 26 fruits.	Number of localities.
1. *Acrothecium lunatum*	4	2
2. *Ascomycete* spp.	3	2
3. *Aspergillus niger*	1	1
4. *Aspergillus* sp.	1	1
5. *Botryodiplodia theobromae*	8	5
6. *Cladosporium herbarum*	18	6
7. *Clasterosporium maidicum*	8	4
8. *Colletotrichum gloeosporioides*	1	1
9. *Coprinus* sp. and other Basidiomycetes	7	5
10. *Eidemia* sp.	4	1
11. *Fusarium ? expansum*	15	6
12. *Fusarium lateritium*	3	3
13. *Fusarium sambucinum*	12	5
14. *Hormodendrum cladosporioides*	3	1
15. *Nigrospora oryzae*	8	3
16. *Oospora* sp.	3	2
17. *Papularia sphaerosperma*	4	2
18. *Penicillium italicum*	13	6
19. *Penicillium* sp.	2	1
20. *Pestalozzia leprogena*	22	6
21. *Phomopsis citri*	3	3
22. *Pseudoplea trifolii*	2	2
23. *Rhizopus*, *Mucor* spp.	9	4
24. *Saccharomyces* sp.	2	1
25. *Trichoderma lignorum*	13	5

III. EXPERIMENTAL METHODS

Grapefruit collected from six localities were brought to the laboratory in sterile containers and surface washings cultured in Petri dishes on malt extract agar in order to identify the fungi present on the skin. Surface sterilization was effected by washing with a saturated solution of borax, steeping in 0·1 per cent. mercuric chloride solution for 5 minutes, and finally washing with sterile distilled water. Surface washings of fruit treated in this manner were found to be free from viable fungal spores. In order to determine the presence or absence, and the nature of latent infections in the fruit, hand sections were then cut from the rind and placed on 2 per cent. malt extract agar. From such material organisms grew out readily and were established in pure culture on potato sucrose agar. Later this technique was suitably modified to determine the depth of penetration of latent infections and their position on the fruit. The surface area of each fruit tested was 1 square inch, or approximately 2 per cent. of the whole. In some instances the area selected yielded upwards of fifty latent infections. It is evident, therefore, that the total number present in a given fruit may be considerable. The tables in Part IV show the presence or absence of any organism and the number isolated from the sample taken. A similar technique was used for papaw and mango fruits.

IV. RESULTS

As anticipated, a considerable number of different fungi was isolated from the surface washings, many occurring consistently irrespective of locality. The assemblage of organisms found on grapefruit during these investigations (Table I) is strikingly similar to that associated with banana diseases by Wardlaw (1935), and observed on Gramineae by Bunting (1927) in the Gold Coast, (Table II). It is reasonable to assume, therefore, that these fungi are commonly present in tropical plantations. It is to be noted that the common citrus pathogen, *Penicillium digitatum*, though frequently observed on fallen fruits in the field, was not among the fungi isolated from skin washings.

Latent infections present in grapefruit at the time of picking are shown in Table III. From this table it would appear that three, or possibly four, organisms are constantly present. Of these, *Phomopsis citri* and *Colletotrichum gloeosporioides* are well-known storage pathogens. *Glomerella cingulata* has been described as the perfect stage of *Colletotrichum gloeosporioides*. The fungus, tentatively described as *Glomerella* sp., is still under investigation. The significance of the Fusarium species has yet to be determined. It will be noted from Table I that spores of these four organisms were seldom found on the outside of the fruit during the period of the investigation.

Using the same technique, papaws and mangoes were also examined for latent infections. *Glomerella* sp. and *Colletotrichum gloeosporioides* were found in both; a Phomopsis species closely allied to, or possibly identical with *Phomopsis citri*, was found in papaws and mangoes.

Having demonstrated the presence of latent infections in full-grown fruit, investigations are now being undertaken to determine at what stage in the development of the fruit they are established. Allowing for climatic factors it may be anticipated that some variation will be encountered. The data already obtained indicate that in some instances they may be established very early.

TABLE II

Fungi Isolated from Different Fruits

Fungi.	Recorded on Grapefruit.	Mango.	Banana (Wardlaw).	Gramineae Gold Coast (Bunting).
Acrothecium lunatum	+	+	+	+
Ascomycete spp.	+	−	−	−
Aspergillus niger	+	+	+	−
Aspergillus sp.	+	−	+	−
Botryodiplodia theobromae	+	−	+	+
Cladosporium herbarum	+	+	+	−
Clasterosporium maidicum	+	+	+	+
Colletotrichum gloeosporioides	+	+	+	−
Coprinus sp. and other Basidiomycetes	+	+	−	−
Dothiorella ribis	+	+	+	−
Eidemia sp.	+	−	+	−
Fusarium ? expansum	+	−	−	−
Fusarium lateritium	+	+	−	−
Fusarium sambucinum	+	−	+	−
Helminthosporium sp.	−	+	−	−
Hormodendrum cladosporioides	+	−	+	−
Mucor spp.	+	+	+	−
Nigrospora oryzae	+	+	+	+
Oospora sp.	+	−	−	−
Papularia sphaerosperma	+	−	+	+
Penicillium digitatum	+	+	−	−
Penicillium italicum	+	+	−	−
Pestalozzia leprogena	+	+	+	+
Phomopsis (citri)	+	+	+	−
Phytophthora palmivora	+	−	−	−
Phytophthora parasitica	+	−	−	−
Pseudoplea trifolii	+	−	+	−
Rhizopus nigricans	+	+	+	−
Saccharomyces sp.	+	+	−	−
Spegazzinia ornata	−	+	−	−
Trichoderma lignorum	+	+	+	−

TABLE III

Grapefruit, Latent Infections

Organism.	No. of fruit examined (90 fruits).	No. of localities.	Total No. of isolations from 90 fruits.
1. *Colletotrichum gloeosporioides* and *Glomerella cingulata*	54	7	448
2. *Fusarium ? expansum*	14	6	29
3. *Glomerella* sp.	52	7	296
4. *Phomopsis citri*	32	7	68

Table IV shows the latent infections found in four size series of 'Julie' mangoes from different trees.

TABLE IV

Latent Infections in the 'Julie' Mango

Weight in gm.	*Glomerella* sp.	*Colletotrichum gloeosporioides.*	*Phomopsis* sp.	*Dothiorella ribis.*
2	—	—	—	—
4	—	—	—	—
8	3	—	—	—
17	1	1	—	—
71	3	—	1	—
81	—	5	—	—
107	3	6	—	—
133	5	—	1	—
179	—	18	—	—
187	1	15	1	—
276	3	16	—	1
6	1	—	—	—
16	1	—	1	—
86	3	—	1	1
160	—	—	2	1
184	1	5	—	6
296	1	2	—	—
315	1	7	—	—
11	1	—	—	—
26	1	4	—	—
35	—	—	—	—
53	—	—	—	—
85	2	2	—	—
128	—	6	1	—
255	—	16	—	—
304	1	21	2	—
2	—	—	—	—
9	—	—	—	—
15	3	—	1	—
25	—	—	—	—
46	—	—	—	—
54	1	—	2	—
86	2	—	—	—
156	—	1	—	—
185	3	16	1	—
311	5	13	—	—

SUMMARY

1. The common storage pathogens of certain tropical fruits may be divided into two groups according to the time and method of their entry into the fruit. These are (*a*) latent infections established during development, and (*b*) wound parasites which usually gain access to the tissues during harvesting and storage operations.

2. By plating out surface washings from fruits it has been shown that the spores of a considerable number of different organisms are abundantly present.

3. Some of these germinate and become established as latent infections within the epidermis, their subsequent development being arrested until the fruit reaches a certain stage of senescence.

4. Other spores, which remain viable on the surface of the fruit, are liable to cause rotting later, provided they are afforded a means of ingress through wounding or disruption of the epidermis.

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The Morphology and Cytology of Typhula Trifolii Rostr.

BY

MARY NOBLE, B.Sc., Ph.D.

(From the Mycological Department, University of Edinburgh)

With Plates II and III, and seven Figures in the Text.

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INTRODUCTION

THIS fungus was described by Rostrup (1902) as a parasite of *Trifolium pratense*, *T. repens*, and of *Medicago lupulina*. Small dark-coloured sclerotia are formed as the resting stage of the fungus, and these are so similar to seeds of Brassica that they may often be regarded as such when they occur in clover seed. Rostrup found them mixed with clover seed from the following sources: east Prussia, Moravia, Hungary, and Norway, and he considered that the disease was likely to be fairly common but unrecognized.

It has been recorded more recently by seed-testers. Dorph-Petersen (1931) reported that, in one season, 10 samples of clover seed out of 860 examined contained the sclerotia; Jorstad (1930) found the fungus in seeds from Norway, and it has once been recorded from Holland by Doyer (1930).

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After some experiments had been carried out with single-spore cultures of *Typhula Trifolii* it soon became apparent that the fungus was heterothallic. Although heterothallism had been shown to exist in the Zygomycetes by Blakeslee in 1904, it was not until some years later that the same phenomenon was demonstrated in the Hymenomycetes. In 1918 Bensaude published an account of sexuality in the Basidiomycetes, proving by means of single-spore cultures that *Coprinus fimetarius* was heterothallic and showing for the first time the significance of the correlation between conjugate nuclear division and clamp connexions. At the same time Kniep (1919–20), in Germany, was studying the cytology of certain Basidiomycetes with a view to determining the origin of the first pair of conjugate nuclei. Although he came to the same conclusion as Bensaude the latter had priority.

While these authors discovered that the first pair of conjugate nuclei owed its origin to the fusion of two hyphae, neither observed such a fusion. This, however, was successfully accomplished by Lehfeldt (1923), who was working under Kniep and who published a detailed account of the origin of the conjugate nuclei in two fungi, *Corticium serum* and *Typhula erythropus*. In Typhula Lehfeldt remarked that the behaviour of the nuclei was not such as had been anticipated by Bensaude and Kniep for Coprinus, although he stated that Coprinus probably behaved more like Typhula than Corticium. The sexuality of Coprinus was studied by Brunswick (1924) and Oort (1929), but these writers dealt with the aspect of the compatibility of strains and not the actual mechanism concerned in the transference of nuclei from one mycelium to another. Then Buller, in 1931, published an account of experimental work with Coprinus, in the course of which he introduced the new term 'diploidization'; and, in interpreting his results, stated that though he accepted Lehfeldt's conclusions, he considered that their confirmation by further work would be desirable. Again, after further investigation (1933), Buller stated that until Lehfeldt's work was confirmed he preferred to believe that the facts were in accordance with a theory which he, Buller, expounded, and repeated that some, at least, of Lehfeldt's conclusions required verification.

Since Lehfeldt was working with a heterothallic species of Typhula, *Typhula Trifolii*, being also heterothallic, appeared to offer a suitable subject for study with a view to the confirmation or otherwise of Lehfeldt's work.

MATERIAL

The material was obtained in the form of sclerotia which were sent over from Poland where they had been found mixed with clover seed.

MICROTECHNIQUE

Material for microtome sections was usually fixed in Flemming's weaker solution. Navashin's fluid was almost as satisfactory as Flemming's, while acetic alcohol and chrom-acetic acid tended to distort the tissues. Sections of the hymenophore were cut at a thickness of 2 μ and those of the sclerotium at 5 μ or 7 μ. As a nuclear stain for microtomed material Breinl's triple stain was used almost exclusively.

Kniep's agar film technique, as modified by Sass (1929), was used for the study of the vegetative hyphae both in the living condition and after fixing and staining. This technique was further modified in order that the hyphae could be examined microscopically without undue external contamination. This called for the provision of a chamber such as is used for the germination of spores in hanging-drop cultures, but large enough to carry the microscope slide of 3 in. by 1 in. on which the agar film was borne (Pl. II, Fig. 8).

Experiments were made with strips of glass fixed together at the corners to form a rectangle. This was placed in a Petri dish and sterilized by dry heat. A sterile slide was then dipped in nutrient agar and placed, film side down, on the glass 'box' for which it formed the lid. The agar film was now ready for inoculation.

The glass boxes were, however, unsatisfactory, as no substance could be found to fix the corners in such a way as to remain sufficiently firm after sterilization. Brass boxes were then made; these were slightly wider than the slide and exactly the same length. Thus the slide rested on the end pieces, which were recessed, and lay between the side pieces so that only the ends of the film were in contact with the brass.

The composition of the medium which was used for the films was as follows: malt extract 2 per cent., gelatin 2 per cent., agar 0·5 per cent., and water 95.5 per cent. This was filtered in the Koch sterilizer, tubed, and replaced in the sterilizer for thirty minutes. As films made with this medium dry up very quickly the foot of the Petri dish was covered with damp blotting-paper from which a rectangle had been cut so as to form a damp chamber within the 'box' and also allow light to be transmitted. The blotting-paper was moistened with sterile water, but it was found that if excess water was present large globules formed on the surface of the film.

It was necessary to alter the depth of the film according to the inoculum and the nature of the resulting mycelium. If only the early stages of spore germination were required a very thin film was used, so that the hyphae grew more or less in the same plane. If, however, two monospore strains were grown together in order to produce a diploid mycelium a thicker film was used, so that the medium would not become exhausted too quickly. The films were allowed to dry down slightly before fixing, and the fixing fluid was that recommended by Sass. Two stains were used with this micro-technique. One was Breinl's triple stain and the other Heidenhain's iron-alum-haematoxylin with light green or malachite green as counter-stain.

THE MYCELIUM

In the following description of the mycelium the distinction of types is solely a matter of convenience; the various 'types' actually merge into one another, making accurate definition impracticable. The terms 'submerged' and 'superficial' will be used as follows:

Submerged. In any culture the mycelium which first appears is usually of the submerged type. It is closely adpressed to the medium or embedded in it and has a hyaline appearance. This mycelium grows out rapidly in a radial direction around the inoculum.

Superficial. The superficial type usually appears after the submerged. Its most characteristic feature is that it is not closely adpressed to the culture medium. While in the submerged mycelium the majority of the hyphae grow along the surface of the medium and some penetrate below it; in the superficial type a few hyphae grow along the surface, but the majority are raised above it. The raised part of the mycelium appears in three forms; it may be white and flocculose, white and granular, or brown and crust-like.

These two types of mycelia, submerged and superficial, may each be composed of either haploid hyphae or diploid hyphae.

The descriptions of the mycelia are arranged as follows:

A. Haploid mycelium. (1) Submerged type, p. 70.
(2) Superficial type, p. 71.
B. Diploid mycelium. (1) Submerged type, p. 72.
(2) Superficial type, p. 72.

A. *Haploid mycelium.*

(1) *Submerged type.* The macroscopic appearance of this mycelium is indicated in the definition of the submerged type of mycelium. The type of branching is an important microscopic feature. The terminal cell usually puts out a branch, but this may be delayed until it becomes the subterminal or even the third cell from the apex. The branches are apparently of unlimited growth and as they, in turn, subdivide, the mycelium spreads very quickly over, and through, the substrate. Branches which arise near the apex form an acute angle with the parent hypha. It appears that the submerged mycelia, both haploid and diploid, spread over the substrate much more quickly than the superficial mycelia. This may be ascribed to the two facts that the branches given off by the apical cells are of unlimited growth and that they form an acute angle with the parent hypha. Branches which arise from the older cells usually form a right angle with the parent hypha, do not grow so long as those which arise from the younger cells, and are often involved in anastomosis. Each cell of the haploid mycelium is typically uninucleate, but cells containing two or even three nuclei occur, especially near the apices of the hyphae.

From observations made on *Typhula Trifolii* it appears that in this species, when two hyphae of the same haploid mycelium anastomose, the nucleus of the one cell usually passes over to the other. It will be shown later that similar movement of nuclei occurs in the diploid hyphae on anastomosis, again where there is no question of diploidization. It is very difficult to determine whether, in the case of nuclear migration from one haploid cell to another, both nuclei persist. Normal cells with two nuclei are not uncommon, so that in a binucleate cell which has been involved in anastomosis, the origin of the nuclei cannot easily be determined. Anastomosis between compatible haploid strains and the consequent diploidization will be discussed later.

Oidia. Oidia are formed on the haploid submerged mycelium. They are very small, measuring approximately 3·6 μ by 0·8 μ and they are colourless. The cells of hyphae bearing oidia are much shorter than those of ordinary hyphae (Pl. III, Fig. 28). The oidia are usually borne in little clusters at the end of short side branches. Each oidium contains one nucleus, but in the cells of the hyphae which bear the oidia the nuclei are not clearly differentiated.

(2) *Superficial type.* The most important difference between the submerged and the superficial types of mycelia lies in the type of branching. In the superficial mycelium the branches are short and abundant. They are, however, relatively longer in the flocculose form than in the granular or crust-like forms of the mycelium.

The flocculose form is usually the first of the superficial mycelia to appear in culture. It is formed at first round the inoculum and then spreads out over the submerged mycelium (Pl. II, Fig. 2 *sup.m.*). When subcultured this flocculose, superficial mycelium gives rise to the submerged mycelium. While the hyphae of the flocculose form together compose a tangled network owing to the frequent and irregular branching, the interhyphal spaces are greater than in the other two forms.

The granular form is similar to the flocculose form, but owing to the cells being shorter, the hyphae are more closely interwoven. The granular form may arise within the flocculose or it may arise independently (Pl. II, Fig. 13 *gr.*). The little granules which make up this mycelium are actually the fundaments of sclerotia. The hyphae in these granules are very strongly branched and lobed, and the cells in this form of mycelium are often swollen and distorted in shape, especially in the hyphae which later form the inner part of the sclerotium. A few of the granules, or fundaments, become much larger and develop into the brown sclerotia.

The brown crust-like form often appears under dry conditions in culture and it is formed of hyphae exactly similar to those which make up the outer rind of the sclerotium. It is suggested that this is a diffuse sclerotium, formed where the conditions are unfavourable for the building up of a true sclerotium. This is further borne out by the occurrence of small abnormal fructifications which develop on the crust.

The cytology of the superficial mycelium presents an interesting point in that many of the short branches do not contain a nucleus. It has been observed that, in the submerged as well as in the superficial mycelium, a branch is not provided with a nucleus until its length is equal to approximately four or six times its breadth. The granular type of mycelium contains a great number of these short branches without nuclei. This mycelium, since it forms the centre of the sclerotium, functions as a reservoir of food-material for the fructification which develops later. This function is fulfilled by virtue of the numerous short branches which contain a great amount of protoplasm within a small space.

B. *Diploid mycelium.*

(1) *Submerged type.* Two important features of this mycelium are: (1) each cell usually contains two nuclei, and (2) the presence of clamp connexions. Clamp connexions will be described later under a separate heading.

The branches are of the same type as in the haploid mycelium, they are long and regular in form, and leave the parent hypha at an acute angle. These long branches arise on the terminal cells of the hyphae. As in the haploid mycelium the branches do not contain nuclei until they are relatively elongated, then the conjugate pair of nuclei from the parent cell enter the base of the branch and divide to form a clamp connexion. According to the method of formation of the clamp connexion the parent cell may be temporarily uninucleate or the parent cell and the branch may each receive a pair of conjugate nuclei more or less simultaneously. A branch may arise from a clamp connexion itself so that two clamps are formed on top of one another.

Although the cells of the diploid mycelium are typically binucleate, cells with three or even four nuclei occur, while occasionally a uninucleate cell with plain septa is found. This suggests that the members of a conjugate pair of nuclei can divide independently even where there is no question of diploidization.

Branches which arise a few cells back from the apex do not form an acute angle with the parent hypha but usually come off at approximately right angles. These branches are comparatively short and frequently anastomose with one another or with the cells of the parent hyphae. After anastomosis is complete it appears that the nuclei of one of the two cells thus connected pass over to the other cell. The nuclei of the cell which is thus invaded gradually disintegrate, sometimes even before the invading nuclei have entered the cell. Bensaude (1918, p. 61) also recorded this phenomenon as occurring in Coprinus.

The nuclei in the submerged mycelium are usually near the middle of the cell, and while the members of the conjugate pairs of nuclei are sometimes closely associated they are more often relatively far apart. Thus when a branch arises one nucleus may pass into it while the other member of the pair is some distance away in the parent hypha. This point will be referred to again in the description of the superficial mycelium.

(2) *Superficial type.* Three forms of the diploid superficial mycelium occur and they are, macroscopically, exactly similar to the forms occurring in the haploid mycelium, namely, flocculose, granular, and crust-like. As in the submerged mycelium the only points of distinction between the haploid and the diploid are the number of nuclei in each cell and the presence of clamp connexions.

Branches of limited growth and irregular shape are very numerous and the cells of these branches are usually shorter and wider than those of the submerged mycelium. The nuclei of the superficial mycelium are often abnormal in shape and there is sometimes only one in each cell.

The separation of conjugate nuclei may be caused by the formation of several branches on one cell. There must be some force which causes a nucleus to enter a young branch, and it has been noted that in the diploid mycelium one member of a conjugate pair may enter a branch while the other member of the pair is some distance away in the parent hypha. If, in such a case, a second branch should arise near the second nucleus it is reasonable to suppose that the nuclei will separate farther and each enter a branch. However, a second factor is involved, the force which holds together the two conjugate nuclei, and it apparently depends on the relative strength of the two forces whether the nuclei separate or remain conjugate.

A branch which contains one nucleus is capable of giving rise to a haploid mycelium. This is borne out by the appearance of oidia in an agar film which had been inoculated with a diploid mycelium. The oidia were exactly similar to those which typically occur on haploid mycelia, and they were actually borne on haploid cells. This points to the conclusion that the diploid mycelia may revert to the haploid condition. Although a small haploid mycelium may thus arise within a diploid it is unlikely that it will become extensive, as it is reconverted to the diploid condition by means of anastomoses and nuclear migrations.

It has been shown that nuclear migrations occur to a limited extent in the typical haploid mycelium and in the typical diploid in circumstances where there is no question of diploidization. It seems probable that the anastomoses which lead to the diploidization of a whole mycelium originate in the same way, but the compatibility of the nuclei thus fortuitously brought together promotes more extensive migration.

CLAMP CONNEXIONS

There are three phenomena of importance in the formation of a clamp connexion: (1) the division of the nuclei; (2) the formation of the septa between the daughter nuclei, and (3) the fusion of the hook with the parent hypha. These phenomena have no constant time relationship to one another, except, of course, that the septa never appear before nuclear division.

Although three types of clamps are described below, there are actually many variations, depending on the various time relationships of the three phenomena. Bensaude described two types of clamp connexions which correspond to the first two types given below.

The majority of the clamp connexions are formed in the following manner. A small protuberance appears on the cell wall and grows backward away from the apex of the hypha to form the hook. If the clamp is produced on the terminal cell of a hypha it is formed about the middle of the cell, but in the case of a branch it appears at the junction of the branch with the parent hypha. The conjugate nuclei now approach the hook, either together or severally, one moving into the base of the hook while the other remains in the parent hypha. This stage is illustrated in Pl. III, Fig. 15, where it is seen that the nucleus moving into the hook is very much elongated. The chromatic nucleolus, discussed on p. 12, appears as a darkly stained streak while the rest of the nucleus which lags behind in the parent hypha does not stain so heavily. Conjugate division now occurs, the spindles lying more or less obliquely, one in the parent hypha and one partly in the hook (Pl. III, Fig. 16); septa are formed between the daughter nuclei and, after some delay, the clamp connexion is completed by the fusion of the hook with the parent hypha and the entrance of the second nucleus into the subterminal cell (Pl. III, Fig. 19). Although the septa are usually formed before the fusion of the hook with the parent hypha their formation may be delayed until after. In Pl. III, Fig. 17, the nucleus in passing into the parent hypha appears to be constricted, as though it were passing through a small aperture. This suggests that the walls between the hook and the parent hypha are only partially broken down so that fusion is not yet complete. In this type of clamp connexion the terminal cell receives its pair of conjugate nuclei as soon as division is completed, but the subterminal cell is temporarily uninucleate until the fusion of the hook allows the second nucleus to enter. This will be referred to as the first type of clamp connexion.

In the second type of clamp, the hook grows round and fuses with the parent hypha before the completion of conjugate division, and in some cases even before the beginning of division (Pl. III, Fig. 20). In this type of clamp the spindles are more or less parallel to one another, one lying completely in the hook (Pl. III, Figs. 21 and 22). Immediately after the completion of division the terminal and the subterminal cells each receive a pair of conjugate nuclei and the clamp is completed by the formation of septa as in the first type. It might be noted at this point that to the observer watching the formation of clamp connexions in living, unstained hyphae there is only one point of distinction between these two types; in the first the septa usually appear before the fusion of the hook with the parent hypha, in the second they always appear after fusion. The exact determination of these two types is, therefore, only possible in stained material.

A third type is found rather rarely in Typhula (Pl. III, Fig. 26). Here the hook, instead of curving round to meet the parent hypha, continues to grow backward so that, except for the direction of growth, it has the appearance of a small branch. A bridging hypha is then formed between the hook and the parent hypha. The behaviour of the nuclei and the time of formation of the septa in this type of clamp are not yet known, but on account of its general structure it may be regarded as rather primitive.

In these cases of conjugate division in the clamp connexion the members of the conjugate pair of nuclei do not usually divide absolutely simultaneously;

the nucleus in the parent hypha divides before the one in the clamp. The time of formation of the septa also varies greatly. If this occurs soon after division the septum in the parent hypha usually appears first, nuclear division in this hypha preceding that in the hook as mentioned above. The nucleoli behave similarly in all types of clamp connexions. They appear to be cast off from the nucleus just before division occurs, and as division proceeds they become smaller, finally disappearing altogether.

It has already been noted that the hook of the clamp connexion grows backward from the apex of the parent hypha and that, in the first type, the subterminal cell alternates between the uninucleate and the binucleate conditions. If the hook had grown towards the apex of the hypha then the terminal cell would suffer this variation in nuclear content. This, however, might be detrimental to growth as the terminal cell sometimes branches before the clamp connexion is completed (Pl. III, Fig. 27). The backward growth of the hook therefore allows the nuclei of the terminal cell to be reconstituted and capable of division before this condition is brought about in the subterminal cell which is not so intimately concerned with the growth of the hypha.

Buller (1933, p. 48) considers that in Coprinus the backward growth is necessary for the maintenance of a constant nucleo-plasmic ratio in the terminal cell. But it appears that this ratio is often upset, both in haploid and diploid hyphae, without any detrimental effect on growth. Haploid hyphae in Typhula often have binucleate terminal cells which are not twice as large as those containing one nucleus and Bensaude also records that, in Coprinus, 'On en trouve beaucoup (de cellules) qui presentent 2, 3, ou même 4 noyaux, surtout aux extrémités en voie de croissance active' (p. 45). In the diploid hyphae of Typhula cells are also found with three and even four nuclei; on the other hand, when two diploid cells anastomose one pair of nuclei disintegrates (p. 72); thus it does not appear that the nucleo-plasmic ratio is so important in this matter as Buller maintains. It is, however, obviously necessary that the nuclei of the terminal cell should be able to divide conjugately without delay.

In this respect the second type of clamp is more efficient than the first. Since the hook has fused before the completion of nuclear division, and the spindles are parallel to one another, the nuclei of the subterminal and the terminal cells are reconstituted more or less simultaneously. The third type, however, may be regarded as least efficient in this respect, since the subterminal cell probably remains uninucleate until the formation of the bridging hypha between the parent cell and the hook.

Buller, (1933, p. 48) in describing the formation of clamps in *Coprinus lagopus*, states that he always observed the formation of the septa before the fusion of the hook with the parent hypha and considers that Bensaude's second method of clamp connexion formation was therefore incorrectly conceived, but he does not give any other cytological evidence in favour of this conclusion. It has been shown, however, that in *Typhula Trifolii* the clamps of the second type are formed according to Bensaude's second type and Macdonald (1934) records that in *Typhula gyrans* the formation of septa is often delayed until after the fusion.

In support of his contention that all clamps are formed according to the first type, Buller refers to work by Harder (1927) on Pholiota. Harder microdissected a clamp connexion, apparently of the first type, before the fusion of the hook, and by isolating the temporarily uninucleate subterminal cell, which does not occur in the clamps of the second type, produced a haploid mycelium. Buller considers that since the 'temporary uninucleate condition of the penultimate cell was a *sine qua non* for producing haploid mycelia artificially', this is evidence that the second type of clamp does not occur. It is difficult to follow the logic of this argument, since, although it does show that in *some* clamps in Pholiota the septa are formed before the fusion, it does not follow that *all* clamps are formed according to this method. The occurrence of Bensaude's second type of clamp connexion has been demonstrated in *Typhula Trifolii*, and in the absence of more conclusive proof to the contrary it may be assumed that it also occurs in Coprinus.

Although clamp connexions have been known for many years their function is still a question of debate. One of the more modern theories is enunciated by Buller, who ascribes a physiological function to the clamp, that of facilitating the passage of protoplasm through the hyphae. Buller prefaces this hypothesis with the statement that 'in both haploid and diploid mycelia each septum has a small open pore through which protoplasm can flow from cell to cell'. If this statement is well founded it follows that in the clamp connexion two pores are present instead of the one in a plain septum; therefore the clamp facilitates the passage of protoplasm. The presence of these pores is evidently closely correlated with the manner in which the septa are formed. Buller, in reviewing the literature on the subject states, 'The first to notice pits in fungus walls was de Bary, in his well-known text-book published in 1884, but he gives no indication whether the pits were closed or open', and again, 'In 1883, Wahrlich . . . demonstrated that septal pores and protoplasmic bridges between cells are characteristic of fungi in general.'

Buller does not mention Strasburger's work entitled 'Zellbildung und Zellteilung', of which the third edition was published in 1880. In this work Strasburger gives a detailed account of the formation of septa in *Saprolegnia.* He describes two distinct methods by which this takes place. In hyphae which are filled with protoplasm the septum appears simultaneously over the whole cross surface of the cell. If, however, the cell is not completely filled with protoplasm then the septum, which Strasburger differentiates into two parts, cell-plate and cell-membrane, appears as a ring which develops centripetally from the outer wall. Strasburger follows this description with the statement (p. 222), 'Ich glaube überhaupt, dass der Theilungsvorgang, wie wir ihn bei *Saprolegnia* geschildert, für alle Pilzhyphen Geltung haben wird, stets in den beiden Modificationen; dass bei vorhandenem Lumen eine von aussen nach innen fortschreitende, bei fehlendem Lumen simultane Ausbildung der Zellplatten vorliegen wird.'

These observations were confirmed and elaborated by Baum (1900), and Bensaude also refers to two types of septa, one which formed 'simultaneously', as in Strasburger's first method and the other forming centripetally as in Strasburger's second.

Buller does not state that he has actually observed either the centripetal formation of the septa of a clamp connexion or the streaming of protoplasm through the pores as he has in many fungi with plain septa. The only detailed drawing he gives of a clamp connexion with central pores appears to be that reproduced from Wahrlich in vol. v, p. 96. Neither the literature quoted by Buller or his own work detract from the apparent value of Strasburger's original observations. Therefore there is evidence that, since the septa of a clamp connexions are laid down in young hyphae filled with protoplasm, they are formed according to Strasburger's 'simultaneous' method in which no pore occurs; and sufficient evidence has not been offered to substantiate the assertion that pores are always present in the septa of the higher fungi.

If the only function of the clamp connexion were to provide two passage ways for the streaming of protoplasm 'through the fruit-body tissues and into the basidia and other hymenial elements' (vol. v, p. 40), it is rather inconsistent that 'in the pilei of many Agaricaceae, even in species in which the diploid mycelium produces clamp connexions . . . clamp connexions are not formed when the nuclei divide conjugately' (vol. v, p. 38). The pileus is surely a region in which such a method of facilitating the passage of protoplasm would be present.

As a clamp connexion is closely associated with conjugate nuclear division it is reasonable to assume that there is some significance in the correlation. It is a feature of conjugate division in any fungus that the nuclei lie side by side and the spindles are more or less parallel to one another, so that after division the conjugate pairs of daughter nuclei are brought together while the sister nuclei are separated from one another.

In all but one of the clamp connexions illustrated the dividing nuclei appear as thin lines, but in Pl. III, Fig. 23, they are shown in the form of spindles. The spindles each occupy a fairly large proportion of the space available in the hypha and this suggests a function of the clamp connexion. By forming the hook in which one spindle is contained it allows the two spindles to be formed side by side and parallel to one another. In a wide hypha with small nuclei the spindles can thus be accommodated within the width of a single hypha and a plain septum is formed after division; in the ordinary vegetative hyphae of such fungi as Typhula, in which the hyphae are relatively narrow and the nuclei large, this is apparently not practicable and the formation of the clamp connexion is necessary. However, the hyphae which compose the stalk of the fructifications of Typhula are wider than those of the ordinary mycelium and there the septa are plain, no clamp connexions are formed.

This also explains the absence of clamps in the pilei of the Agaricaceae where the cells are wider than in the vegetative hyphae. While in the Uredineae the absence of clamps is accounted for by the fact that in this group the nuclei are consistently smaller in comparison to the hyphae than those of the Hymenomycetes. This function of the clamp connexion was suggested by Bensaude and has more lately been adopted by Bessey (1935).

It is a matter of common observation that in many fungi plain septa are formed on hyphae which also bear clamp connexions. These plain septa are associated with two forms of nuclear division: independent division of conjugate nuclei, and conjugate division in which the two spindles are accommodated side by side within the single hypha. In most fungi the second alternative appears to occur more frequently than the first.

THE NUCLEI

The nuclei in this fungus are small and very difficult to stain. The appearance of the nucleus at rest, stained with iron-alum-haematoxylin, is shown in Pl. II, Fig. 19. There is a spherical, black part and a more or less oval part which does not stain so deeply and appears to be more granular than the other. These two parts are usually close together, but occasionally they are separated, and while they may lie side by side they are more often one behind the other, as is shown in the figure. The nucleus sometimes becomes elongated and in circumstances which indicate that it is moving, as in Pl. III, Fig. 15, it assumes the form of a streak of dark staining matter, while the lighter staining part is not visible.

Only a few instances of nuclear division have been observed and most of these were in the clamp connexions. Some stages of conjugate division are shown in Pl. III, Figs. 15, 16, 21, 23, and 24. In Fig. 15 the black spherical part of the nucleus is elongated and appears to be moving into the hook, and as it stains very deeply it may be regarded as chromatic. Some of this chromatin is later cast off from each dividing nucleus and appears as two small spherical objects which eventually disintegrate (Pl. III, Fig. 16 at *n.n.*). During division the more granular, lighter staining part of the normal nucleus is no longer visible and it appears to be entirely absorbed in the formation of the chromatic figure. The spindle usually appears as a line, but in some instances it assumes the form of a typical spindle (Pl. III, Fig. 23).

The spherical black part of the normal nucleus is probably a chromatic nucleolus, but there is not sufficient evidence to allow of the exact determination of the nature of these nuclei and the changes which they undergo during division.

MULTISPORE CULTURES

The mycelium which is first formed in multispore cultures is of the submerged type. If the inoculum consists of many spores of different strains the mycelium is at first haploid, but rapidly changes to the diploid condition.

This change is only apparent microscopically. The superficial mycelium may then arise, when it assumes any or all of the three forms already described (p. 72): flocculose, granular, or crust-like.

The sclerotia are then formed. Their size and number are considerably affected by temperature. At low temperatures, about 13° C., many large sclerotia are formed, while at about 23° C. their formation is stopped altogether. Another factor in this matter is the nature of the substratum. If the medium is deep, and of a suitable nature, the sclerotia are formed freely; on the other hand, if there is no great depth of medium only a few sclerotia appear. If the medium is very thin and dry the brown crust-like mycelium is formed. Oat agar seems to be a suitable medium for the formation of the brown crust, and masses of fused sclerotia are also formed on this medium.

The fructifications appear almost immediately after the sclerotia are mature, but if the latter are removed from culture and planted in soil or sand a resting period is usually necessary before the fructifications are formed. Fertile fructifications thus formed are usually normal in size and shape. In only one case has a fertile fructification appeared in culture. This specimen became branched at the apex, a phenomenon which often occurs in culture.

The fungus grows well on malt or oat agar, and satisfactory growth is also obtained on a medium composed of an extract of clover seed stiffened with agar. Similar media made with beans or with beet-root were also used. On soil extract agar the fungus grows very slowly and long, very slender sterile fructifications are formed. As is noted in dealing with haploid fructifications the fungus is only able to grow for a short time on a medium composed of agar solution alone.

DIPLOID FRUCTIFICATIONS AND SCLEROTIA

A normal diploid fructification, obtained by germinating a sclerotium in soil, is illustrated in Pl. II, Fig. 3. The average length of such fructifications is 1–1·5 cm., the largest being 1·7 and the smallest 0·8 cm. The stalk is usually clearly defined, but in some specimens it may only be distinguished from the hymenium by the absence of spores. Hairs occur at the base of the stalk and immediately below the hymenium. In one case a band of hairs occurred in the middle of the stalk (Pl. II, Fig. 4). This was due to the external conditions becoming unfavourable at the time when the basidia were about to be developed, resulting in the cessation of growth and the appearance of hairs in place of basidia. On the fructification being removed to more favourable conditions growth was renewed and the hymenium subsequently developed, the sterile hairs remaining as a collar around the stalk. The stalk, in normal specimens, swells out rather sharply to form the hymenium which entirely covers the upper part of the fructification.

In the case of conditions remaining unfavourable for some time, the developing fructification may more or less wither; if, for instance, the substrate is allowed to dry up then the fructification, instead of remaining upright, becomes prostrate. However, on conditions becoming more favourable again another fructification may arise from the stalk of the withered one and grow up vertically in the normal way.

The stalk and the central core of the hymenium are composed of diploid hyphae whose cells are shorter and wider than those of ordinary vegetative hyphae, and these cells do not bear clamp connexions. Owing to the tangled growth of the hyphae immediately below the basidia it is difficult to determine whether clamps are present in this region or not; so far they have not been found. Clamps are present, however, on the narrow hyphae which cause the downy appearance of the base of the stalk and hymenium.

The cytological phenomena of the basidium appear to follow the usual course. A pair of conjugate nuclei enter the base of the basidium, become closely associated with one another, and fuse to form the fusion nucleus which is comparatively large. This nucleus divides twice, giving rise to four daughter nuclei one of which passes out to each of the four spores. Owing to the very small size of the nuclei in this fungus it has not been possible to study the stages of the divisions in detail.

The average spore size is 10·9 μ by 4·9 μ. The length varies from 8·6 μ to 12·4 μ, and the breadth from 3·8 μ to 6·4 μ.

The sclerotia. These are small dark brown or black bodies whose diameter varies from 1 to 2 mm. (Pl. II, Fig. 2). The outer wall, or rind, is quite hard in mature sclerotia and has a rough outer surface. The formation of this rind has been described by Macdonald (1934) in his work on *Typhula gyrans*, which in this respect appears to be very similar to *Typhula Trifolii.*

When a sclerotium is cut across the rind is seen to be relatively thin, while the bulk of the sclerotium consists of a mass of white hyphae, very closely interwoven (Pl. II, Fig. 5). Large sclerotia are often hollow. The rind is continuous, but is slightly indented at one point, the little cavity formed by the indented part being filled with the thin-walled hyphae which previously attached the sclerotium to the substrate. This feature adds considerably to the difficulty of identifying the sclerotia in samples of seed since it resembles the hilum of a Brassica seed.

MONOSPORE CULTURES

Monospore cultures may be divided into two classes, those which produce sclerotia and fructifications and those which do not. 'F' and 'G' belong to the first class and 'K', 'S', and 'N' to the second.

All these strains were isolated by spreading spores on a thin layer of filtered malt agar, punching out small pieces of agar containing single spores, and transferring to fresh culture medium. 'F' and 'G' were thus isolated early in 1933 and they still (May 1936) retain certain features which distinguish them from one another. 'F' is a typical example of the first class of monospore culture. When subcultured the mycelium grows out fairly rapidly, and as it never forms the superficial type of mycelium the culture always presents a flat unbroken surface. The microscopic appearance of 'F' is also characteristic. The cells are very regular in size and the branches are typical of the submerged type of mycelium, long and very regularly formed. The edge of the colony of 'F' is smooth and oidia are freely formed.

The strain 'G' is not quite so typical of the first class as 'F', since it may produce the brown 'superficial' hyphae which are often formed in monospore cultures of the second class. In cultures of 'G' the colony is formed very slowly and has quite a characteristic appearance; instead of covering the surface of the medium, as 'F' does, it forms a little raised colony often with radial indentations. The young outer part of the colony is usually light yellow while the inner part, which is older and more raised up, is light to dark brown. The colony as a whole forms a compact, tough mass.

Microscopically, 'G' is again quite different from 'F'; the hyphae are more irregular in form and they grow more slowly, while oidia are not formed freely. As 'F' and 'G' have been in culture for an equally long period and have been treated in exactly the same way it appears that these characters must be regarded as individual variations.

In the strains 'K', 'S', and 'N' which form fructifications, the mycelium, which is first formed, is submerged, i.e. the type which is constantly present in 'F' and 'G'. This grows out quickly and is followed by the granular or flocculose type of superficial mycelium. The sclerotia and fructifications are formed exactly in the same way as in the diploid cultures. When monospore strains of this type are grown on agar films haploid fructifications sporing vigorously may appear as soon as seven days after the inoculation was made, while small sclerotia may also be formed on agar films.

It has not yet been determined whether there are two 'sexes' or four in *Typhula Trifolii* as the results of a sufficient number of cross inoculations are not yet available. Up to the present, however, it appears that strains of different sex cannot be distinguished morphologically.

HAPLOID FRUCTIFICATIONS AND SCLEROTIA

The most striking feature of a typical haploid fructification of *Typhula Trifolii* is its small size (Pl. II, Fig. 8). Specimens have been obtained in culture measuring 1·5 mm. in length. As these fructifications were sporing vigorously their small size cannot entirely, if at all, be ascribed to immaturity. These specimens were, however, exceptional, as the average size of the fructification in culture is about 5 to 7 mm., i.e. about the size of a very small diploid fructification.

While diploid fructifications never attain their normal size or shape in artificial culture the reverse is true of the haploids. Thus the two types of fructification may not be very different in culture as regards size. However, when the sclerotia of each type, haploid and diploid, are germinated in soil or sand the difference between the resulting fructifications is marked, the diploids being at least three times as large as the haploids.

In culture the haploid fructifications appear as miniatures of normal diploids. The stalk is slender and swells out at the top to form the hymenium. The shape of the basidium appears to resemble very closely that of a diploid fructification, while the four basidiospores are borne on very slender sterigmata. One feature of the typical diploid is not found in the haploid fructification. The stalk, instead of bearing hairs at the top and bottom, is either downy all over or more or less glabrous. When hairs occur, however, they are rather longer at the top and bottom of the stalk than in the middle.

When dealing with diploids (p. 79), it was mentioned that, under favourable conditions, young fructifications can arise from the stalk of a withered specimen. The same is true of haploid fructifications. Other exceptional specimens are those in which several seem to have fused together or which may be regarded as abnormally large specimens tending to branch at the apex. One such fructification is shown in Pl. II, Fig. 7; it was 8 mm. in length and at the time of photographing was sporing actively.

In fructifications which arise directly from the culture medium, without having a sclerotium at the base, the basal part of the stalk is often dark brown (Pl. II, Fig. 6). The colour is darkest below and gradually shades off into the normal yellowish-white of the upper part, the brown part consisting of thick-walled hyphae such as make up the outer layer of the sclerotium. It appears as if in some way these thick-walled hyphae are substituted for the sclerotium.

As in the diploid fructification the basidium carries four spores, each of which is borne on a slender sterigma, all being proportionally smaller than in the diploid. The spores are hyaline and ovate, with one side not so rounded as the other, the less rounded side facing inward on the basidium. The spores are smaller than those formed on the diploid fructification, measuring approximately 5·1 μ by 2·6 μ. This figure was obtained by measuring spores which had been smeared on a slide prepared with egg albumin, fixing at once in Flemming's weaker solution and staining with Heidenhain's iron-alum-haematoxylin. Spores treated in exactly the same way, but taken from diploid fructifications, measured approximately 10·5 μ by 4 μ; it is therefore improbable that the small size of the spores is due to this treatment. This method was adopted as it was very difficult to measure such spores accurately except by staining and using an oil immersion objective.

The spores are viable and have now been grown through several generations; although the later generations grow more slowly than the first they still produce many fructifications which spore vigorously. Like the spores from diploid fructifications they are uninucleate. They germinate quite readily on culture medium and produce very slender germ tubes which later attain the diameter of the typical haploid hyphae. No oidia have yet been found on the mycelia producing 'haploid' fructifications, but as only relatively few strains have been studied this may not be a constant feature. Actually the basidiospores have become the equivalent of oidia since they are all of the same sex or strain.

It is interesting to note that the nature of haploid fructifications varies from one fungus to another. Hanna (1928) working with *Coprinus lagopus* found that diploid fruit-bodies were more vigorous and spored more quickly and more regularly than haploid fruit-bodies, even the most highly developed haploids producing but relatively few spores. Hanna ascribed this lack of vigour to the presence of one nucleus in each of the cells of the haploids in contrast to the pair of conjugate nuclei in each cell of the diploids. The haploid fructifications of *Typhula Trifolii*, however, although they are smaller than the diploids are certainly not less vigorous.

Another important point of difference between the haploid fructifications of Typhula and those of Coprinus is in the size of the spores. Hanna recorded that in Coprinus the spores which are formed on haploid fructifications do not differ in size from those borne on diploid fructifications; it has been shown that, in Typhula, they are smaller.

Haploid sclerotia. These are, in shape, structure, and colour, very similar to diploid sclerotia. As in the fructifications there are two important differences between the haploid and the diploid. The haploid sclerotium is composed of haploid hyphae and it is smaller than the diploid.

The haploid fructifications and sclerotia are, in Typhula, very similar to the corresponding diploid structures, but they differ in size and in the nature of the hyphae with which they are constructed. In dealing with Coprinus, Hanna considered that the haploid condition of the hyphae is reflected in the diminished vigour of the haploid fructifications; in Typhula it appears that vigour remains as great in the haploid as in the diploid, but the size is considerably reduced.

HETEROTHALLISM

In reviewing the literature concerning heterothallism it was shown that Lehfeldt was the only investigator to describe the mechanism by which the nuclei of one mycelium pass into another mycelium and cause the formation of clamp connexions and conjugate nuclei.

It is now proposed to summarize Lehfeldt's work, particularly that dealing with *Typhula erythropus*. This will be followed by an account of the results obtained in the present investigation of *Typhula Trifolii*. Lehfeldt's results, as well as those of the present investigation, were obtained by the use of the agar film technique. In Lehfeldt's case, however, the slides could not be examined so conveniently before staining as in the modified form of the technique described on p. 69 of this paper.[1]

Lehfeldt's work was principally concerned with the two fungi *Corticium serum* and *Typhula erythropus*. In Corticium when quite young germ tubes come into contact with one another they anastomose. It is usually the apices which so fuse. The nuclei in these cells then approach one another and divide conjugately to form the first clamp connexion. Thus, in Corticium, there is a close correlation between the passing of the nucleus from one hypha to another and the formation of the first clamp connexion. In *Typhula erythropus* it appears that, for some unknown reason, the germ tubes are unable to anastomose as in Corticium, so that the hyphal fusions and transference of nuclei are delayed until the mycelia are older. In *Typhula* there is, almost without exception, no correlation, either in time or space, between the point of anastomosis and the first clamp connexion. The few exceptions were found in anastomosis between young cells. 'Es wäre denkbar, dass hier nur alles so glatt vonstatten ging, weil es junge Zellen, nahe der Fadenspitze, waren, die von der Anastomose betroffen wurden' (p. 50).

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[1] Considerable difficulty has been experienced in elucidating Lehfeldt's conclusions, and it appears that this difficulty has not been confined to the author alone. As the paper is of considerable importance, extensive quotations have been used so that there shall be no doubt as to the source of the interpretation given here.

In *Typhula erythropus*, when two compatible hyphae come into contact with one another fusion may be accomplished by means of a short bridging hypha; Lehfeldt does not state whether the bridge is always present or whether the hyphae may fuse directly. A nucleus from one hypha now passes to the other leaving behind an empty cell. The invading nucleus divides, one of the daughter nuclei may move towards the apex of the hypha while the other may move towards the base. After such movement, during which the invading nuclei frequently divide, the septa of the invaded hypha are found to be very irregular in that some are partially broken down and other new septa are formed, sometimes close beside the broken ones. It is considered that partial or complete dissolution of the septa is correlated with the migration of the nuclei. 'Ich halte sie für später gebildet, als die halbe Wand schon durchbrochen war und einem Kerndurchtritt Raum gegeben hatte' (p. 43). Such partially dissolved walls are not always easily distinguished from whole walls: 'Wenn der fehlende Teil solcher halben Wand genau bäuchlings oder genau rücklings liegt, mag man sie leicht für volle Querwände halten, bis ein Vergleich aller Tiefeneinstellungen mit der Mikrometerschraube erweist, dass doch ein kleines Stück fehlt' (pp. 42–3). Rawitscher (1914), to whom Lehfeldt refers, had previously described similar migration of nuclei through 'half-walls' in *Ustilago Maydis*. It may be mentioned at this point that, in a recent paper, Lamb (1935) has recorded the occurrence of 'half-walls' in *Puccinia Phragmitis*, where he states 'there is fairly good evidence that a certain amount of septal dissolution does occur, very much after the fashion originally described by Lehfeldt'. Lehfeldt has also shown that the side wall opposite such a half-wall is often bulged out as if by the passing nucleus.

The invading nuclei will have now reached the younger parts of the hyphae, the apices of the main hypha, and its branches. The nuclei then form conjugate pairs with some of the indigenous nuclei, and when these conjugate pairs divide, the first clamp connexions appear. Lehfeldt states that in *Typhula erythropus* there is no great difference in length between haploid and diploid cells, and that when a haploid mycelium is converted into a diploid some of the indigenous nuclei degenerate, and are replaced by division of the conjugate nuclei.

If a hypha which possesses both kinds of nuclei, invading and indigenous, should anastomose with another hypha of the same mycelium which has not yet received invading nuclei, then those pass from the former to the latter. This occurs even if the former hypha does not yet bear clamp connexions. It is further stated that when two hyphae are involved in anastomosis, the transference of nuclei is eventually mutual, both hyphae being converted to the diploid state.

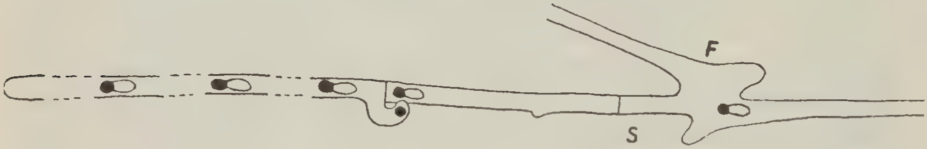
Lehfeldt has shown that there is a close correlation in *Corticium serum* between the anastomosis and the first clamp connexion resulting from it; in Typhula, on the other hand, as the result of anastomosis several clamps may appear simultaneously on the hyphae at some distance from one another. 'Dass zwischen *Corticium serum* und *Typhula erythropus* in der Anlage der ersten Schnalle in Wechselbeziehung zur ersten Anastomose ein grosser Unterschied besteht, glaube ich erwiesen zu haben. *Coprinus fimetarius* steht in seinem Verhalten bei diesen Vorgängen vermutlich der Typhula viel näher, und der Umstand, dass auch Kniep bei *Corticium varians* und *Colybia conigena* gleichfalls keine einfachen Verhältnisse vorfand, lässt schliessen, dass auch diese beiden Pilze, bei denen nach meinen Ergebnissen Heterothallie nicht ausgeschlossen ist, obwohl der zytologische Befund zunächst auf Homothallie deutet, sich mehr dem Typhula-Typus nähern. Identisch ist ihr Verhalten mit Typhula allerdings auch nicht, denn die halben Querwände und Ausbauchungen scheinen zu fehlen, die leeren Zellen scheinen zum mindesten erheblich seltener zu sein' (pp. 50–1). This quotation is important, since *Coprinus fimetarius* is synonymous with *Coprinus lagopus*, with which Buller later carried out his researches on heterothallism.

In interpreting instances of anastomosis and diploidization in *Typhula Trifolii* many technical difficulties are encountered. One of the greatest, and one which is always present, is due to the fact that clamp connexions are not formed immediately two compatible haploid mycelia come into contact with one another; but this is delayed until the mycelia have become fairly closely interwoven, so that it is often impossible to trace the origin of the hyphae concerned.

Then, also, a dividing nucleus may appear very similar to one in the course of degeneration. In the early stages of division the nucleolus is cast off and gradually diminishes in size before disappearing altogether, while the chromatin is often very difficult to stain. A nucleus in the course of disintegration is also represented by a nucleolus which diminishes just as in the dividing nucleus. Again, some septa may stain very well while others in the same preparation are scarcely discernible. Bensaude also experienced this irregularity in staining and ascribed it to varying pH values within the mycelium.

It is therefore evident that in the explanations of the origin and destiny of nuclei and septa it is impossible to be dogmatic. There are so many possible explanations that only suggestions can be offered with occasional indications of that which is most likely to be correct. While these examples appear to be incomplete and disjointed, it may be mentioned that they are the result of the examination, both in the living condition and after fixing and staining, of a considerable number of agar film preparations. Moreover, it seems that the complete elucidation of these cytological problems is more likely to be accomplished by this method than by any other.

In this fungus it appears that very young germ tubes are unable to anastomose with one another, so that the first cases of anastomoses and nuclear migration do not occur until the germ tubes are about thirty-six hours old.



TEXT-FIG. 1. Anastomosis and nuclear migration between compatible haploid hyphae.[1]

The first two examples to be described occurred in preparations of germ tubes about forty-eight hours old, and the others in preparations of adult hyphae of compatible strains.

In the first example, illustrated in Text-fig. 1, conditions are relatively simple. Two haploid hyphae have fused at the point marked F. The terminal cell of one hypha has fused with either the terminal or the subterminal of the other. A nucleus has passed over and moved up to the apex of the hypha. Since there are now three nuclei in the apical cell it is probable that this cell was originally binucleate. The invading nucleus became conjugate with one of these indigenous nuclei and divided to form the clamp connexion. The septum at S may have been formed either before or after the passage of the nucleus. It appears to be entire, but may actually be a half-wall of which the missing part was either behind or before the remaining part, in which case it is very difficult to detect. The nucleus at F is probably a daughter nucleus of the invading nucleus, or possibly indigenous.

Young cells are also involved in the second example, illustrated in Text-fig. 2. Fusion between two haploid hyphae appears to have taken place at F, but this is not quite clear in the preparation. The branch AB is without a nucleus, so it is quite probable that the missing nucleus is responsible for the abnormal conditions in the hypha CD. It is not possible to trace the hypha CD any farther back from the apex as it is obscured by other hyphae. The cells towards the apex of CD will now be discussed. The nucleus at N1 is in the course of degeneration, only the nucleolus persisting. It is therefore probable that this is an indigenous nucleus. Of the two nuclei in the next cell, N3 is in the same condition as N1, but N2 is clearly defined and quite normal in shape; therefore N3 is likely to be indigenous and N2 an invading nucleus.

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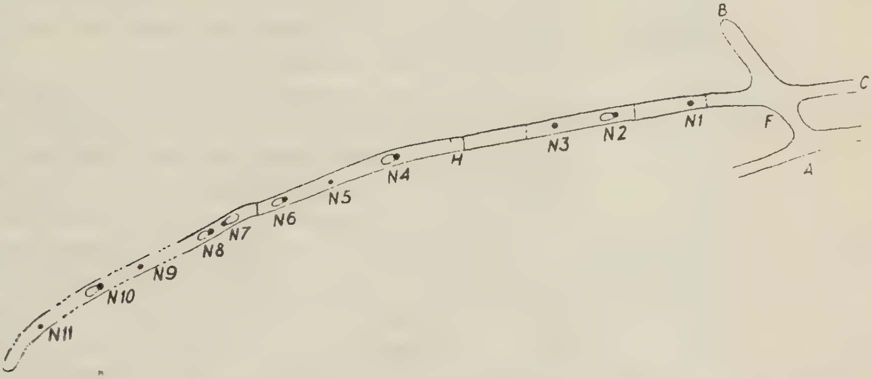
[1] Broken lines in the drawings indicate shortening of the hyphae.

An empty cell then follows. Either the nucleus of this cell has entirely disintegrated or one of the septa is secondary in origin, i.e. it has been formed after the passage of the invading nucleus.

At H there is a fairly well defined half-wall close to an entire wall; the latter is probably secondary in origin. There is a space in the protoplasm on the other side of the whole septum, but it is not clear whether this is merely a vacuole or another half-wall. Of the three nuclei in the next cell, N4 and N6 are normal, but N5 is degenerating. N4 and N6 may be invading nuclei and N5 indigenous.



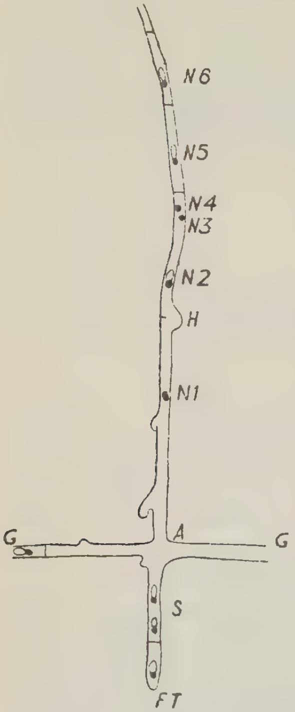
TEXT-FIG. 2. Anastomosis and nuclear migration between compatible haploid hyphae.

The terminal cell contains five nuclei, of which N7, N8, and N10 are normal while N9 and N11 are degenerating. The position of N8 and N7 suggests that they are conjugate. This cell provides a good illustration of the difficulty which may be experienced in accounting for each nucleus in such cases where the normal haploid condition is upset. It is possible that here a septum is completely dissolved and that the five nuclei were originally contained in two cells.

In the third example, illustrated in Text-fig. 3, the hyphae involved are of the two strains 'F' and 'G'. The subterminal cell of 'F' has fused with a comparatively old cell of 'G'. Anastomosis occurred at the point marked A, probably by means of a short bridging hypha which is now hidden by the hypha at A.

A 'G' nucleus evidently passed over into the 'F' hypha. It divided at once, and one of the nuclei in the binucleate cell at S is probably one of the daughter nuclei of this division. The terminal cell FT is as yet undisturbed and contains its original F nucleus. The G nucleus now in the subterminal cell will probably divide and a daughter nucleus will pass into the terminal cell FT, form a conjugate pair with the F nucleus, and on division the first clamp connexion will appear. Another possibility is that the pair of nuclei in the subterminal cell 'S' may divide conjugately and form a clamp connexion.

Another daughter nucleus of the invading G nucleus has moved away from the apex in the F hypha. The nucleus N1 seems to be disintegrating, thus it is probably an F nucleus. A half-wall appears at H accompanied by a bulge on the opposite wall; therefore the G nucleus has probably passed this point. N2 may be either a G or an F nucleus, but since it shows no signs of degeneration is probably a G nucleus, the product of a division which occurred at this point. The other daughter nucleus of this division is either N3 or N4. This is the farthest point reached by the G nuclei, as the cells containing N5 and N6 are undisturbed. The hypha F bears the rudiments of branches, one immediately above the point A and the other just below N1.



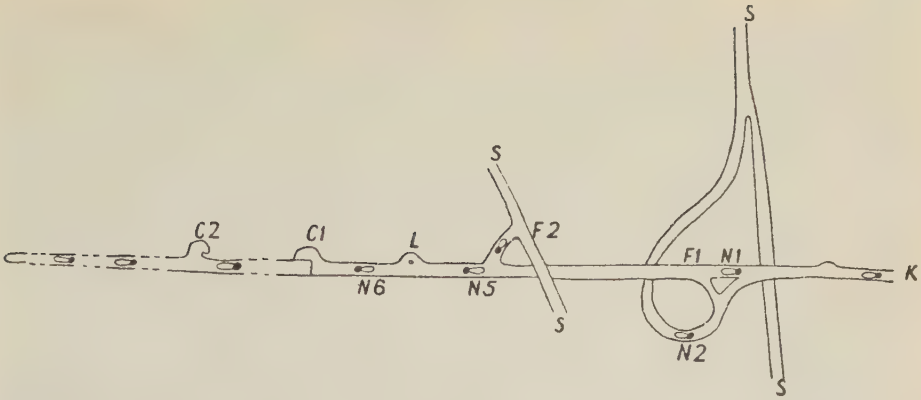
Text-fig. 3. Anastomosis and nuclear migration between compatible haploid hyphae.

In the fourth example, Text-fig. 4, the hyphae involved are of the strains S and K, which are two of the haploid strains capable of producing haploid fructifications. Two points of fusion are visible, two different hyphae of S have fused with one K hypha, at the points F1 and F2. F1 is actually a double fusion, as the apex of S has forked and each branch of the fork has fused with K. There are very few nuclei in the vicinity of F1, which suggests that a nucleus from K may have moved into S or that migration has not yet taken place, and that N1 and N2 are both in their original positions.

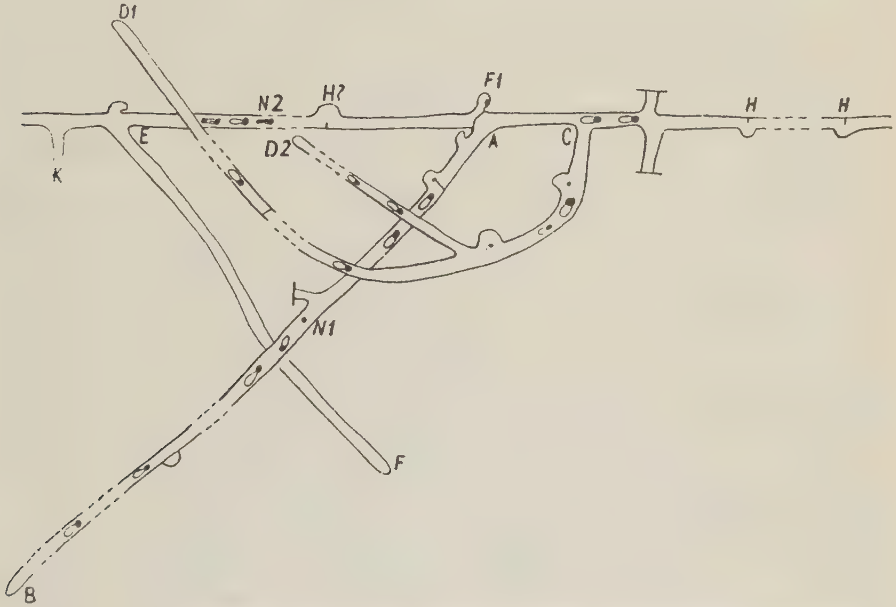
Nuclei have passed from S to K at the second fusion, F2, as clamp connexion formation has begun in the apical cells of K. The structure at L is rather interesting. It appears to be a clamp connexion of the second type, described on p. 74, in which the hook is fully formed and fused with the parent hypha before the division of the nuclei. In this case N5 and N6 will divide conjugately within it. It is rather unusual to find a clamp connexion thus forming in an intercalary position. C1 and C2 are easily recognized clamp connexions; the diploid mycelium is now established.

It is clearly seen in this case that one hypha (the K hypha) may be involved in more than one fusion, so that nuclei from another mycelium may enter at various points and form conjugate nuclei. Or, alternatively, the hypha may receive a nucleus at one point, and at another transfer one of its own nuclei to the other hypha. In the same preparation from which this last example was taken a hyphal branch was found which had fused four times with hyphae of the opposite strain.

The fifth example of fusion between haploid hyphae is illustrated in Text-fig. 5. Again the strains F and G are involved, but the hyphae are rather diffi-



TEXT-FIG. 4. Anastomosis and nuclear migration between compatible haploid hyphae.



TEXT-FIG. 5. Anastomosis and nuclear migration between compatible haploid hyphae.

cult to follow, as they occur deep down in the agar film. The main hypha which is running horizontally cannot be seen on either side of the part shown, as it is obscured by other hyphae.

The hyphae are very confused at the point F1 and it is possible that fusion took place here between the apices of an F and a G hypha. It is unlikely that the main hypha consists entirely of one strain, since it is old and vacuolated on each side of the part shown. This indicates that the fusion took place somewhere near F1. For the present discussion it will be assumed that the fusion occurred at this point.

The hypha AB which has arisen at this point is almost regularly binucleate. As has been stated, the hyphae at the point of origin of this branch are very confused and it is possible that nuclei are present in the cell at A. The next cell, towards the apex from A, has two conjugate nuclei, then a branch occurs. There may be a clamp connexion on the under side of this branch, but as it soon becomes merged in a tangled mass of haploid hyphae, it is possible that the 'branch' is actually a haploid hypha which has anastomosed at this point with the diploid. The nucleus at N1 is very small and apparently is becoming disintegrated. It may be one of the nuclei which were present in the hypha while it was haploid. The terminal and subterminal cells of this hypha AB are typically diploid and will give rise to a diploid mycelium.

The hypha CD1, CD2 is only partially diploid, and this indicates that the invading nucleus, or one of its daughter nuclei, reached this branch later than the branch AB. The basal cell of this hypha bears a clamp connexion, but the nuclei which would normally be present in this cell have moved away from their original position. The nucleus at C is probably one member of the pair, while the other, an 'invading' nucleus, has continued its migration towards H.

Septa are not shown in the clamp connexions in the branch CD1, DC2. They may, however, form later as the formation of septa in clamp connexions is often delayed. This also applies to the hypha AB.

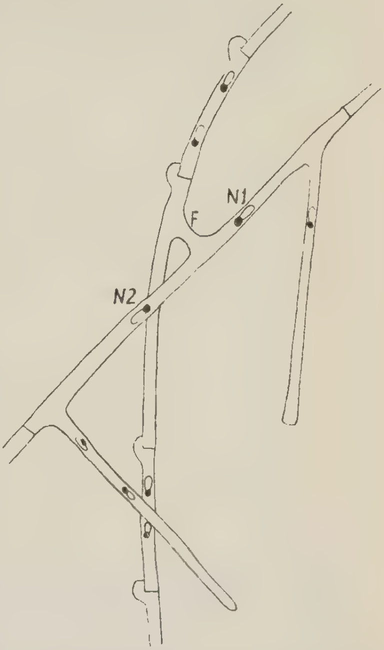
If the fusion actually took place at F1 then this is a case of exchange of nuclei between the two mycelia, since the branch EF has also become diploid. The two nuclei which it contained are not shown in the figure. The bulge on the main hypha at H? may be a half-wall, but as it is seen obliquely it cannot be identified with certainty. There is the possibility that it is a clamp connexion, but the absence of conjugate nuclei in its vicinity is evidence in favour of its being a half-wall. At H, H two very clearly defined half-walls with bulges are shown. They were observed in the living hyphae as little bulges, but the half-walls were not visible. After fixing and staining, however, it was apparent that these structures corresponded exactly to those which Lehfeldt described as occurring in *Typhula erythropus*.

The nuclear matter at N2 may be accounted for in several ways, part of it is likely to be indigenous and part invading, but it is impossible to identify these parts.

Since the branch at K is haploid the invading nucleus has probably still to reach this point, and this further indicates that the transference of nuclei occurred at F1 and that the migration of invading nuclei is proceeding away from that point.

In conclusion, this is a case of anastomosis between the apical cells of two mature hyphae. Mutual transference of nuclei has taken place and the haploid condition of the hyphae has been upset. The diploid condition is, however, now established in the younger cells of the branches, and these cells will eventually give rise to a diploid mycelium.

Two examples of anastomosis between diploid and haploid hyphae will now be briefly described. The first is illustrated in Text-fig. 6. Fusion occurred at the point F, and since the diploid cell is now empty both nuclei have presumably entered the haploid cell. The two nuclei at N1 and N2 are probably the conjugate pair which migrated and which now replace the nucleus originally present in this cell. (It will be recalled that in the case of anastomosis between two diploid cells, the nuclei from one cell pass over to the other while the indigenous nuclei degenerate.) One of the nuclei in the lower diploid branch is probably an invading nucleus, formed by the independent, non-conjugate division of either N1 or N2. On the other hand, both nuclei may be indigenous since binucleate cells are not uncommon in haploid hyphae.



TEXT-FIG. 6. Anastomosis and nuclear migration between compatible diploid and haploid hyphae.

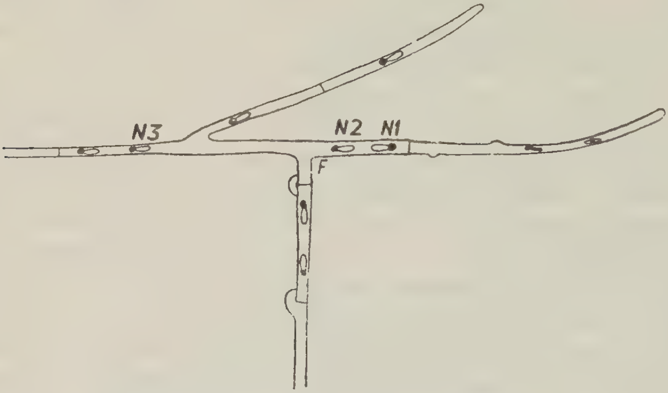
In the other case of anastomosis between a diploid and a haploid hypha, Text-fig. 7, a clamp connexion has been formed at the point of anastomosis, F, and again two conjugate nuclei have entered the haploid cell. The nuclei N1 and N2 appear to be conjugate; either they are both invading nuclei or one of them is indigenous. In the latter case the invading pair must have separated, and it is possible that N3 is the other member of the pair. The invading nuclei will now divide and migrate farther through the haploid hyphae.

Further examples were found in which the originally haploid hyphae had been more completely converted to the diploid condition, but owing to the presence of many other hyphae not concerned in the anastomosis, the original point of fusion could not be traced.

On considering the evidence offered by these, and other cases of anastomosis between two haploid hyphae and between a diploid and a haploid hypha, it appears that the process of anastomosis and subsequent nuclear migration takes place in *Typhula Trifolii* as follows:

Fusion may take place directly between two hyphae, or a small bridging hypha may be formed. Once the hyphae are in open communication with one another the transference of nuclei occurs. In most cases the nucleus of one of the cells passes over to the other, without previous division. It has been observed that this transference of nuclei may not be a matter of independent movement of nuclei through the protoplasm but a violent expulsion of protoplasm which carries the nucleus with it.

The invading nucleus now divides, perhaps once or several times. This is followed by the progression of the daughter nuclei, which may again subdivide, through the hypha, in all directions. To allow of the passage of these nuclei the transverse septa are either partially or wholly dissolved, thus 'half-walls' are formed. The side wall opposite such a half-wall may be bulged out as if by the passing nucleus, and the septa thus broken down may be replaced by others of later origin.



TEXT-FIG. 7. Anastomosis and nuclear migration between compatible diploid and haploid hyphae.

If the anastomosis occurs on young cells the nuclei may become conjugate without extensive migration and the first clamp connexion is formed almost immediately. When older cells are involved in anastomosis there is a period between the first transference of nuclei and the formation of the first clamp connexion during which the cells of the invaded hyphae are disturbed from the equilibrium of the haploid state. As soon as the invading nuclei reach the younger cells they become associated with the indigenous nuclei in pairs, and these hyphae again reach a state of equilibrium, this time in the diploid condition. Although in the first case, when young cells anastomose, clamp connexions appear without extensive migration, this does not mean that such migration may not subsequently take place. The important point is that the clamps appear first and the migration may follow; where older cells are involved migration must precede clamp-connexion formation. In the latter case, of course, young cells may be formed by the branching of the older hyphae.

In the fusion of diploid and haploid hyphae one interesting point is that both the nuclei from the diploid hypha enter the haploid. This is in agreement with Rawitscher's explanation of the 'illegitimate diploidization' which Buller observed in Coprinus. Rawitscher (1933) suggests that when a diploid hypha fuses with a theoretically incompatible haploid, both nuclei from the diploid enter the haploid cell, displace the indigenous nucleus, and continue the growth of the hypha, clamp connexions being formed on their division.

Buller's original suggestion was that only one nucleus passes over to the haploid cell and becomes conjugate with the indigenous nucleus, but Rawitscher's hypothesis is well borne out by these observations on *Typhula Trifolii*.

DISCUSSION

It was stated in the introduction that Bensaude, in 1918, working with *Coprinus fimetarius*, first proved that the phenomenon of heterothallism existed in the Hymenomycetes. Bensaude showed that two single-spore strains of this fungus remained in the haploid condition when grown separately, but formed a diploid mycelium with clamp connexions when brought into contact with one another. She could not, however, discover the exact mechanism by which the change from the haploid to the diploid condition was accomplished, as she states, 'Jamais nous n'avons pu voir d'une façon indubitable la fusion plasmogamique qui serait l'origine du filament secondaire en voie de formation.' Bensaude also gave a description, illustrated by drawings, of the methods of clamp-connexion formation in Coprinus. Kniep had also been publishing papers on the cytology of the Hymenomycetes in the years immediately prior to the publication of Bensaude's work. Owing to the Great War, however, Bensaude was not aware of his work until 1918, while Kniep did not know of Bensaude's paper until some time later. The following sentence appears in a paper which Kniep published in 1919: 'Ich habe schon früher hervorgehoben, dass sich daraus die theoretisch wichtige Möglichkeit ergibt, dass zwei von verschiedenen Myzelindividuen herrührende Kerne das erste Kernpaar bilden.' Although Kniep was thus forestalled by Bensaude, one of his students, Lehfeldt, had already started to work on this question of the origin of the conjugate pair of nuclei. Lehfeldt had a great advantage over Bensaude in using Kniep's agar film technique for observing the hyphae. This technique is very similar to that used in the present work on *Typhula Trifolii*. Lehfeldt showed in his paper the necessity of some such technique in work on this question of anastomosis and nuclear behaviour; in this the writer concurs as regards work on *Typhula Trifolii*; for critical work it is essential that the hyphae should be fixed and stained after examination in the living condition, and even then great care is required in tracing out the hyphae involved in anastomosis.

Lehfeldt was very guarded in his statements concerning the phenomena of anastomosis and nuclear migration in *Typhula erythropus*. He did, however, make it clear that the invading nuclei move from the point of anastomosis to the young cells of the hyphae. If young cells are involved in anastomosis then the nuclei become conjugate and the clamp connexions appear relatively quickly. Then Lehfeldt also pointed out the state of disturbed equilibrium which prevails after anastomosis, and which is followed by restored equilibrium in the diploid condition and the appearance of clamp connexions.

Kniep's death occurred in 1930, and in the same year Buller gave a paper to the International Botanical Congress on work on Coprinus of which a full account is given in the fourth volume of his 'Researches' published in 1931. In this volume a series of experiments is described and the interpretation is largely based on Lehfeldt's work, although it is considered that this work requires confirmation. It will be recalled that Lehfeldt, in reviewing the results which he obtained with Typhula, did not say that Coprinus behaved in exactly the same way, but that in his opinion it probably did behave more like Typhula than Corticium.

Relying on the cytological work of Lehfeldt, and on his own observations, Buller describes (vol. iv, p. 275) the course of the diploidization process in a large haploid mycelium of Coprinus which fuses with a haploid mycelium of opposite sex and receives from it one nucleus. He mentions the division and subdivision of the invading nucleus and the progression of the daughter nuclei through the septa by means of their partial or complete dissolution, and the establishment, in the terminal cells, of conjugate nuclei, followed by the formation of clamp connexions.

So far, this agrees with Lehfeldt's conclusions, except in the omission of the replacement of the broken septa by other plain septa. On considering these phenomena in more detail, however, Buller assumes that the invading nucleus divides the exact number of times required to provide a daughter nucleus for conjugation with each indigenous nucleus encountered during migration, and he describes this method of diploidization as a very simple process. But while this interpretation may be true of Coprinus, Lehfeldt made it clear that in Typhula such simplicity is exceptional and occurs only in young cells. There is no justification in Lehfeldt's work for the impression that the process is equally simple throughout the whole mycelium. In the diagrams of the diploidization process in Coprinus, in vol. iv, the nuclei are very regular in number and the origin of each one is evident. These figures are admittedly diagrammatic, e.g. the openings in the septa are not shown, but the impression is given that the passage of nuclei through the hyphae is a simple and well-regulated process. This does not, however, agree with Lehfeldt's conclusions regarding *Typhula erythropus* or with the results obtained with *Typhula Trifolii*.

Buller gives only one set of drawings of an observed case of diploidization in Coprinus, vol. iv, p. 238. In these drawings the progressive diploidization of the cells of a haploid mycelium is shown. One important point is that the haploid mycelium appears to be non-septate until the appearance of the clamp connexions; and, in fact, no plain septa at all are mentioned in the description or shown in the drawings of the haploid hyphae. In another drawing of haploid hyphae, made under apparently the same conditions as the former, anastomoses between haploid and diploid hyphae are illustrated. In this case plain septa are shown in the haploid hyphae, but they are as yet undisturbed as the diploidization process has not yet begun. A more detailed account of the condition and position of the plain septa and of the clamp connexions would add conviction to the later statement made in vol. v, p. 167, 'When watching haploid hyphae of *Coprinus lagopus* being diploidized I never once observed a bulging of hyphae at a septum which had to be passed by a nucleus, nor did I see any septa disappear. In my volume iv, in discussing the diploidization process, I accepted Lehfeldt's conclusions; but now, as a result of my own observations upon, and a study of the literature upon, the structure of septa in the Higher Fungi, I feel that Lehfeldt's work needs verification before it can be accepted.' Buller then introduces the theory that the nuclei pass through the open pores which, he considers, are present in each septum.

It may well be that where such pores are present the nuclei pass through them; but Lehfeldt's work and that described in the present investigation show that partial and complete dissolution of septa do occur in Typhula, and as the occurrence of half-walls in one of the Rust fungi has recently been described by Lamb (1935), it is clear that such structures are produced in at least two groups of the Basidiomycetes. Further, it was shown in discussing the clamp connexion (p. 76) that the presence of an open pore in each septum is not consistent with the observations of Strasburger and others.

Further comparison might be made between conditions in Coprinus and those in the two species of Typhula under consideration, but the above will indicate sufficiently clearly the complexity of the subject and the necessity for further investigation.

SUMMARY

Typhula Trifolii was obtained from Poland in the form of sclerotia which had been found mixed with clover seed. Using the sclerotia as inocula the fungus was brought into pure culture. Haploid and diploid mycelia are distinguishable only by cytological characters; macroscopically the mycelia are alike. The haploid hyphae have plain septa and usually one nucleus in each cell; the diploid hyphae have clamp connexions at most of the septa and usually one pair of conjugate nuclei in each cell. Anastomosis and migration of nuclei between the two cells thus connected occurs in the haploid and the diploid mycelia even where there is no question of heterothallism or diploidization. Some of the indigenous nuclei in the invaded cells disintegrate.

Monospore cultures usually produce neither sclerotia nor fructifications, but in some these structures are formed and are therefore made up of haploid hyphae. Haploid sclerotia are smaller than diploid, but otherwise very similar. Haploid fructifications are also smaller than diploids, but while the former are fertile in artificial culture the latter are sterile. Spores borne on haploid fructifications are smaller than those borne on diploid fructifications.

Three types of clamp connexions are described. The first two correspond to the two types described by Bensaude as occurring in Coprinus.

Typhula Trifolii is heterothallic; compatible haploid strains have been grown together to form diploid mycelia. The cytology of anastomoses between compatible haploid mycelia, and between haploid and diploid mycelia, has been studied by means of an agar-film technique. When anastomosis

occurs between such mycelia, migration of nuclei takes place, followed by the upsetting of the equilibrium of the cells of the invaded hyphae. Some of the indigenous nuclei of invaded cells disintegrate. Nuclei migrate by means of the partial as well as the complete dissolution of transverse septa. In the former case 'half-walls' are formed, sometimes the side wall at such a partially dissolved septum is bulged out apparently to increase the size of the aperture through which the nucleus passes. When the invading nuclei have reached the younger parts of the hyphae, which may be close to the point of anastomosis or several cells distant, they conjugate with indigenous nuclei, and on the division of these conjugate pairs the clamp connexions appear. Anastomosis between diploid and haploid hyphae has much the same result in causing the migration of nuclei from the former to the latter so that it also becomes diploid.

The work of Lehfeldt on *Typhula erythropus* has been confirmed. A discussion is given on the significance of this confirmation with regard to recent work on the method of diploidization in Coprinus.

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EXPLANATION OF PLATES II AND III

Illustrating Dr. Noble's paper on 'The Morphology and Cytology of *Typhula Trifolii* Rostr.

PLATE II

Fig. 1. Culture of the diploid mycelium. c, crust-like form of the superficial mycelium, forming on the thin, dry parts of the oat agar.

Fig. 2. Typical culture of diploid mycelium on malt agar. *Sc.*, sclerotia; *sub.m.*, submerged mycelium; *sup.m.*, superficial mycelium.

Fig. 3. Typical diploid fructification formed from a sclerotium germinating in soil.

Fig. 4. Diploid fructifications formed from sclerotia germinating in sand. The formation of hairs was due to growth being temporarily stopped.

Fig. 5. Thick sections of typical diploid sclerotia. h, the indented part which represents the 'hilum'.

Fig. 6. Haploid fructifications in culture, arising directly from the substrate, not from sclerotia. *d*, dark-brown hyphae at the base of the stalks of the fructifications; *f*, flocculose form of superficial mycelium.

Fig. 7. Large haploid fructification in culture. The small white colonies seen around the fructification have arisen from spores shed by this fructification.

Fig. 8. Agar-film technique as used in the present investigation. The lid of the Petri dish was removed and the slide turned film side up for the purpose of photography. The two colonies on the agar film are compatible haploids of the second type, i.e. those which produce haploid fructifications, two of which are seen in the colony at the right.

Fig. 9. The compatible haploid strains 'F' and 'G'. *d*, diploid superficial hyphae forming between them. Diploid sclerotia appeared later at *d*.

Fig. 10. Culture of G into which F was introduced at the point *f*, where sterile diploid fructifications now appear.

Fig. 11. Culture of haploid mycelium with crust-like form of the superficial mycelium.

Fig. 12. Monospore culture of the strain 'S' showing haploid sclerotia.

Fig. 13. Culture of diploid mycelium. *fb*, flocculose form of the superficial mycelium; *gr*, granular form of the superficial mycelium.

Fig. 14. *a*, culture of diploid mycelium, and *b*, culture of haploid mycelium made at the same time and grown under the same conditions. The haploid mycelium has produced many fertile fructifications while the diploid mycelium has produced none.

PLATE III

Figs. 15–19. The first type of clamp connexion. Figs. 20 and 21. Clamps which may belong to either the first or second type. Figs. 22–5. Clamp connexions of the second type. Fig. 26. The third type.

Fig. 15. The hook has formed and the nuclei are moving into position.

Fig. 16. Conjugate nuclear division, probably the telophase. The spindles are represented by two thin lines, in an oblique position. *n*, nucleoli in the course of disintegration.

Fig. 17. The entrance of the second nucleus into the subterminal cell. The septa have already formed, and the nucleus appears to be constricted in passing from the hook into the parent cell.

Fig. 18. Conjugate division is almost completed, but the septa are not yet formed.

Fig. 19. A stage which is characteristic of the first type of clamp. The septa are formed, but the second nucleus has not yet entered the subterminal cell.

Fig. 20. The hook is fully formed, but the nuclei are still at rest in the parent hypha.

Fig. 21. The parallel position of the spindles suggests the second type of clamp, but fusion may occur before the completion of nuclear division, in which case the clamp connexion would belong to the first type.

Fig. 22. Conjugate division has not yet begun, but the hook has already fused with the parent hypha.

Fig. 23. Conjugate division with parallel spindles, probably the metaphase stage.

Fig. 24. Later stage than that of Fig. 23. The nucleoli have now almost disappeared.

Fig. 25. The septa have not yet appeared, although the nuclei have more or less regained their normal condition.

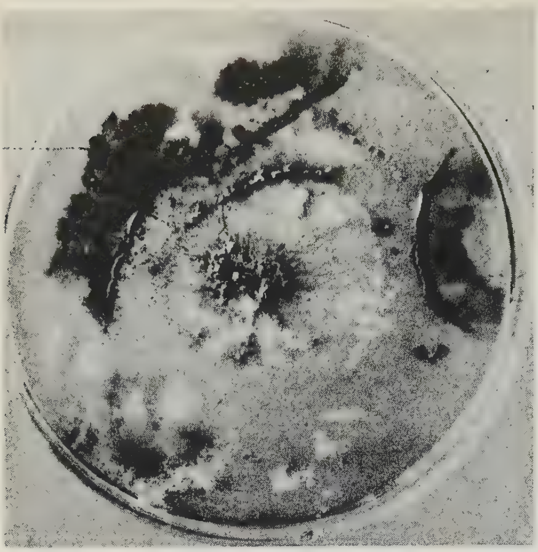
Fig. 26. The branch-like type of clamp connexion.

Fig. 27. Clamp connexion, showing the advantage of the backward direction of growth of the hook. The terminal cell has branched and a nuclear division appears to be imminent, while the subterminal cell is still in the uninucleate condition. Thus conjugate division is not yet possible in the subterminal cell.

Fig. 28. Hyphae bearing oidia.

Fig. 29. Anastomosis between two diploid hyphae. One of the cells thus connected now contains three nuclei and a fourth is also about to enter the cell.

Fig. 30. Later stage in anastomosis between diploid hyphae, N1 and N2, the indigenous nuclei are disintegrating, N3 is about to enter the cell as will also N4.



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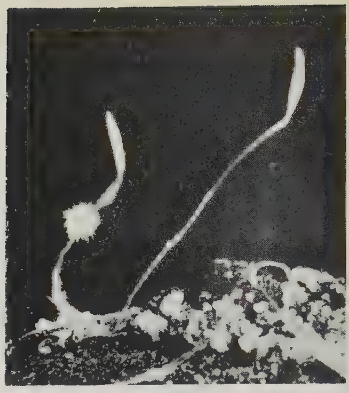

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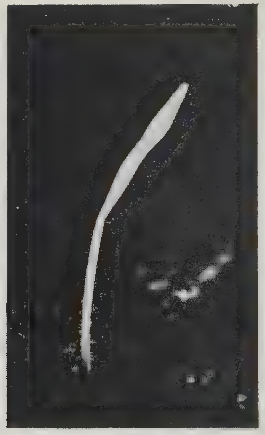

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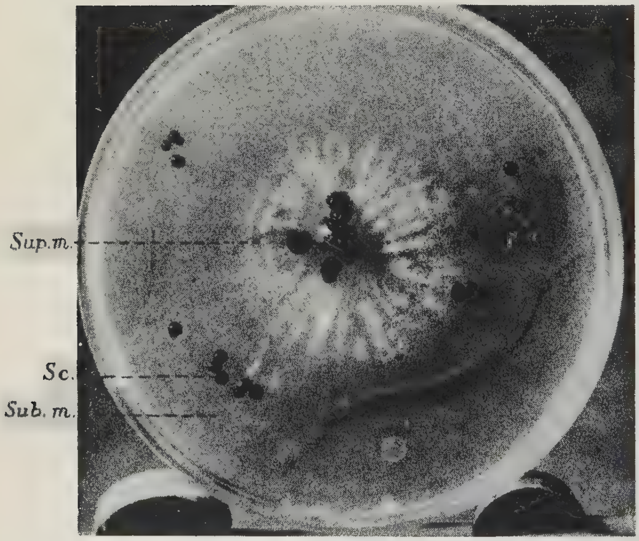

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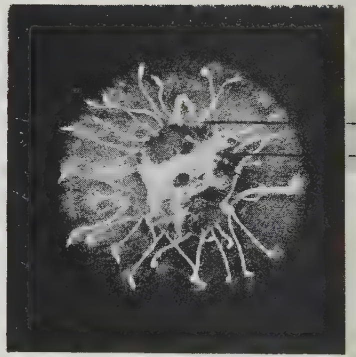

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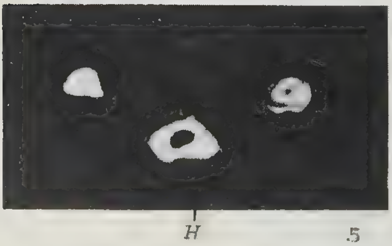

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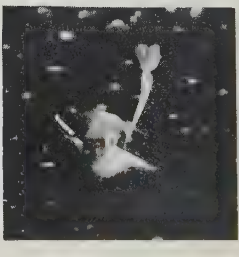

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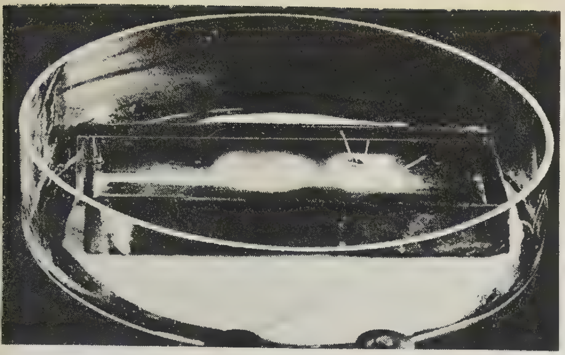
NOBLE — TYPHULA.



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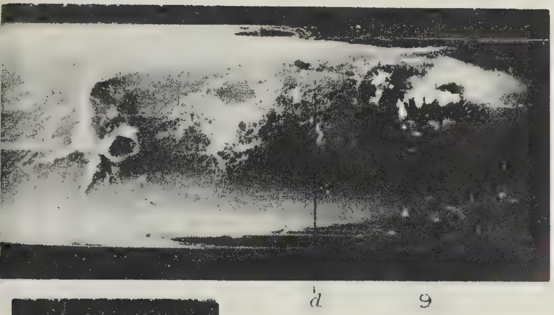

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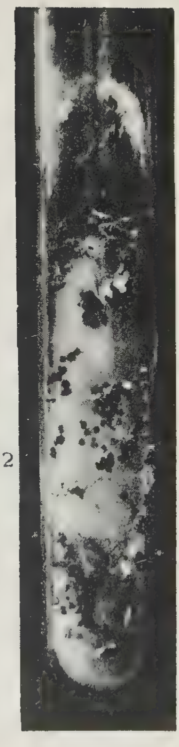

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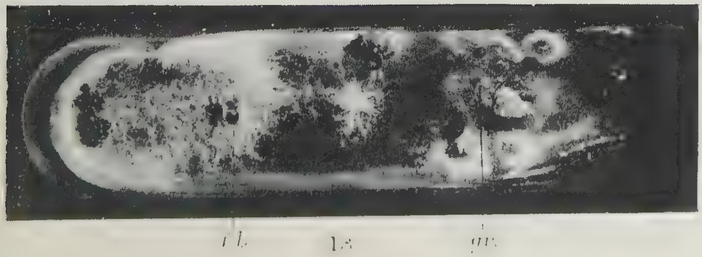

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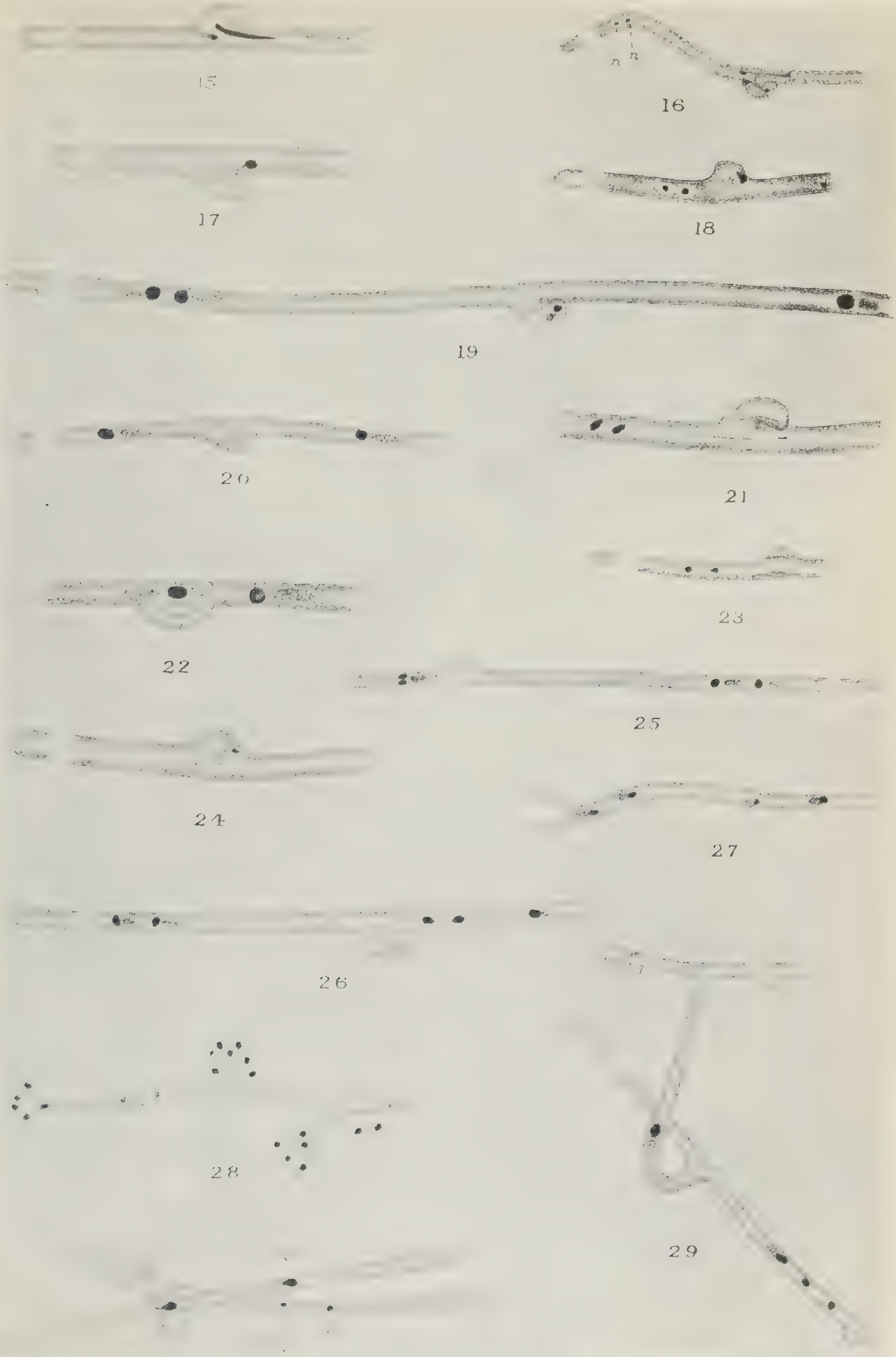

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NOBLE — TYPHULA.

Contributions to the Study of Lachnea melaloma

BY

H. C. I. GWYNNE-VAUGHAN

With fifteen Figures in the Text

ON October 21, 1933, material of *Lachnea melaloma* Alb. et Schwein was collected on burnt ground near Oxshott. The fructifications were reddish to dingy yellow in colour and bore externally brown, septate hyphae with clavate ends. Ascospores, as they were shot out of the asci, were collected on glass slips arranged above the fruits and germinated in about twenty-four hours at 20 to 25° C. Cultures in Petri dishes were made on Barnes' medium with 0·1 per cent. glucose, and on the medium 'M' of Gwynne-Vaughan and Barnes (1930) with glucose, with filter-paper, and with both. They produced oidia but failed to fruit. Mycelia from single spores were then paired and ascocarps obtained in about 50 per cent. of the cultures, showing the fungus to be heterothallic with two compatible or complementary strains, A and B. Later in the investigation a culture was obtained which fruited alone. This fact may be correlated with the occasional appearance in section of binucleate ascospores.

In addition to synthetic media, cultures were made on sheep-dung agar with filter-paper, on oat agar with filter-paper, and on potato agar. On the last named considerable aerial mycelium developed. 'M' with filter-paper and weak oat agar with filter-paper gave the best results.

While fresh spores germinated readily, spores nine months old took some days to germinate.

The material from Oxshott was never very prolific; after being kept in culture for a year it began to show signs of erratic growth. In the autumn of 1934 ascocarps were collected in Sprouston Woods, near Norwich, and at Burnham Beeches. They differed in minor particulars from the Oxshott material; ascospores from Sprouston would germinate only after being heated, and the oidia were borne on dichotomously branching hyphae, so that they appeared in clusters, instead of being formed, as in the Oxshott fungus, by the breaking-up of ordinary filaments. The new material, however, resembled that from Oxshott in forming fruits too sparsely to make hopeful an attempt to trace the full nuclear history, and, owing to pressure of other work the investigation was set aside. The whole of the culture work, as well as part of the microscopic investigation, had been carried out by Mrs. H. S. Williamson, to whose care and judgement the present study owes much, and whose death in December 1934 was a serious loss to British mycology. Though the work

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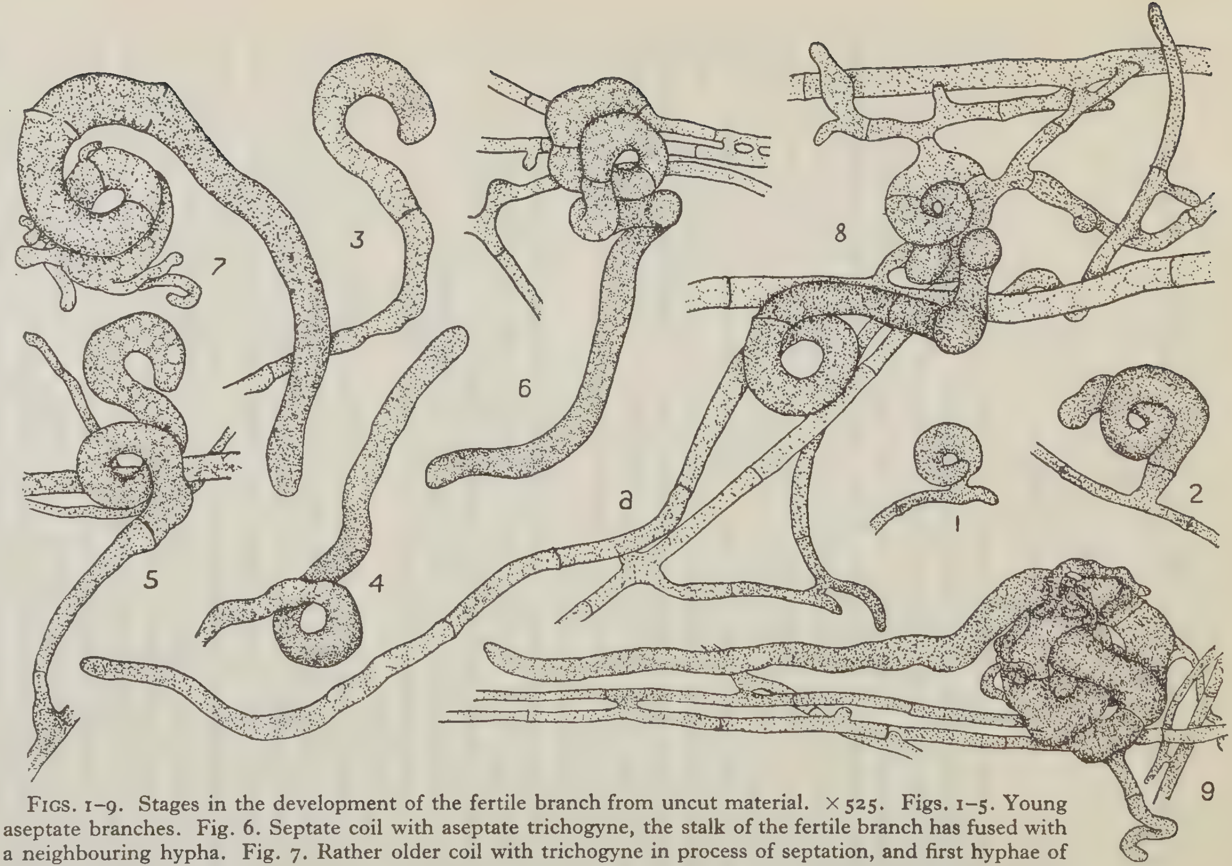


FIGS. 1–9. Stages in the development of the fertile branch from uncut material. × 525. Figs. 1–5. Young aseptate branches. Fig. 6. Septate coil with aseptate trichogyne, the stalk of the fertile branch has fused with a neighbouring hypha. Fig. 7. Rather older coil with trichogyne in process of septation, and first hyphae of sheath. Fig. 8. Fertile branch arising laterally on a wide hypha; outgrowths from the stalk have fused with neighbouring cells, the trichogyne is very long and septate, but the formation of the sheath has not begun, at *a* the trichogyne is in contact with a vegetative hypha. Fig. 9. Coil with developing sheath, mycelial fusions and trichogyne in process of septation.

is incomplete, it appears worth while to place on record the investigations made. Only Oxshott material has been employed for this purpose.

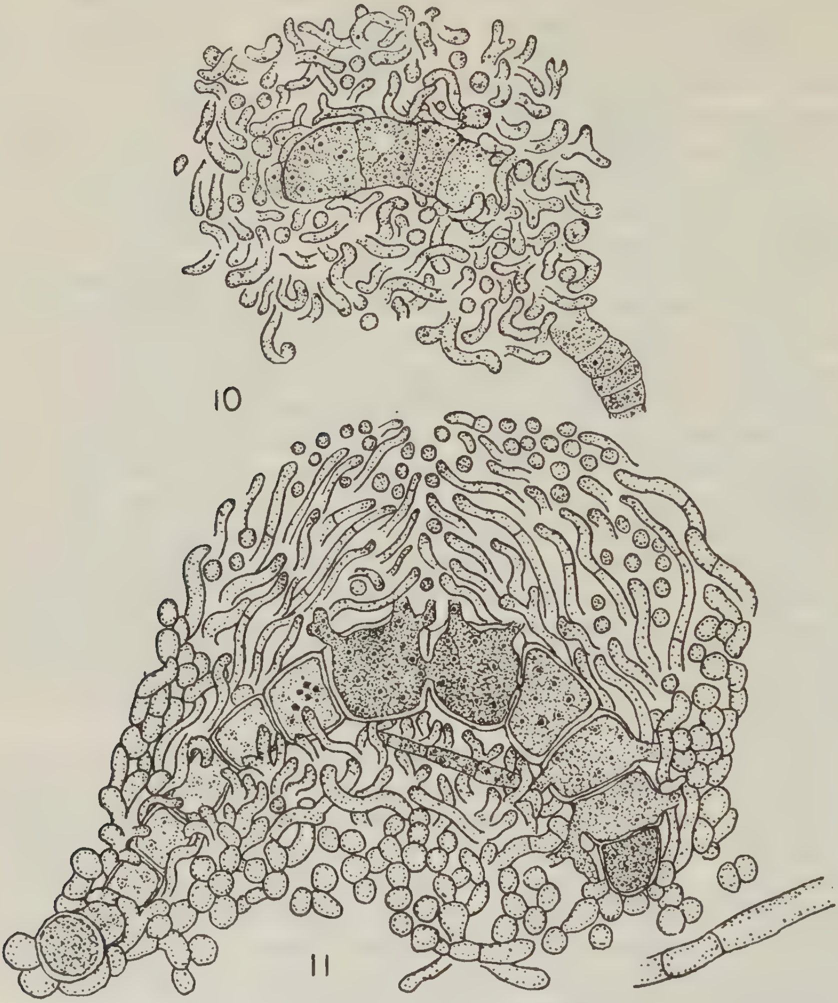
When paired spores are used in Petri dishes ascocarps appear about a week after inoculation. The first evidence of their development is the formation of a thick, recurved primordial branch (Fig. 1) which enlarges, is cut off by a wall and becomes more or less coiled (Figs. 2–5), the free end forming a prolongation which may perhaps be regarded as a trichogyne. This structure is for some time without transverse walls (Figs. 4, 6), septation begins in the proximal region (Figs. 7, 9) and passes towards the tip. At about the same time branches grow from the stalk cells of the coil (Figs. 7, 9) and constitute the beginnings of the sheath.

So far as could be observed, the trichogyne plays no part in development; it was never seen to fuse with vegetative hyphae, with oidia, or with any structure that could be regarded as antheridial. The possibility that a fusion has occurred at *a* in Fig. 8 cannot be altogether excluded, but it is certainly not convincing. On the other hand, mycelial fusions at this stage are common. They may take place between a vegetative hypha and the stalk of the coiled primordial branch (Figs. 6, 9), or between vegetative hyphae and branches from the proximal cells of the coil (Fig. 8). Since ascocarps contain both A and B spores it may be inferred that their primordial coils receive in this way nuclei of the complementary strain. Doubtless in the same way the sheath cells developed from the stalk of the coil contain, as in *Humaria granulata* (Gwynne-Vaughan and Williamson, 1930), both A and B nuclei. The vegetative filaments vary very much in thickness; it is often the narrower branches from which the coils arise (Fig. 6) and to which they form a striking contrast.

After the sheath has grown up additional septation takes place in the coil, the constituent cells (Fig. 10) being as broad or broader than they are long.

During the early development of the sheath the nuclei of the middle cells of the coil increase in size and nuclear fusions are found. The fact that these occur singly (Fig. 10) suggests that they are apogamous, like those in Granulata, and that normal fertilization, characterized by the simultaneous union of many pairs of nuclei (Gwynne-Vaughan and Williamson, 1931, 1932, 1934), no longer takes place. This bears out the evidence from uncut material of the behaviour of the trichogyne.

A little later (Figs. 11, 12), when ascogenous hyphae are beginning to develop, the middle cells of the coil are crowded with dense contents and show numerous large nuclei. Two or more cells are in continuity through wide protoplasmic strands; ascogenous hyphae arise from one (Fig. 12), two (Fig. 11), or possibly more cells. It is difficult to be sure whether branches such as the deeply stained outgrowth just below the cell bearing ascogenous hyphae in Fig. 12 will give rise to asci; more probably, in spite of their well developed nuclei, they are concerned only with the formation of paraphyses. By this time the contents of the trichogyne have begun to disintegrate and are in striking contrast to those of the stalk; so far as could be observed, the transverse septa of the trichogyne are never broken down.



FIGS. 10 and 11. Young ascocarps in section. × 700. Fig. 10. Part of a coil in section surrounded by hyphae of sheath; in the cell on the left two nuclei are in process of fusion. Fig. 11. An older ascocarp in longitudinal section, showing part of the coil, two cells of which are giving rise to ascogenous hyphae, above these the first paraphyses are pushing upwards.

Soon after the middle region of the coil has become enclosed, a number of the investing hyphae turn upwards and inwards (Fig. 11), forming the first paraphyses. Later the outer, protective branches extend beyond them (Fig. 12) and they show a tendency to separate along the middle line, where the hymenium will later appear.

The stage at which ascogenous hyphae arise may vary, those seen in Fig. 11 being as well advanced as those in the older fructification shown in Fig. 12. They branch freely and, while they are still in continuity with the cells of the oogonial region, mitosis takes place both in the oogonial nuclei and in those which have passed out of the coil. Septation of the ascogenous hyphae follows, and development proceeds along the lines described by Gwynne-Vaughan and Williamson (1931, 1932, 1934) in *Pyronema confluens* and other species.

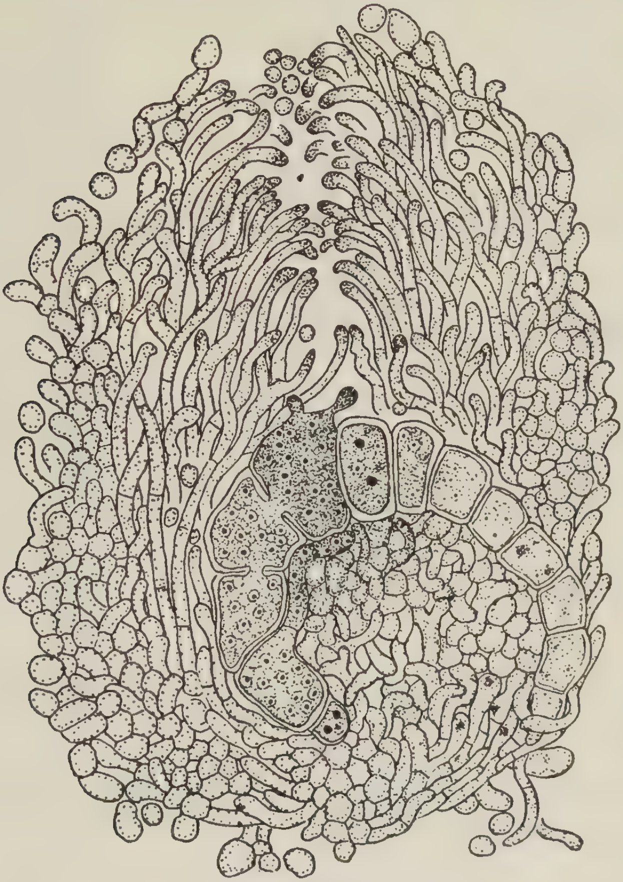
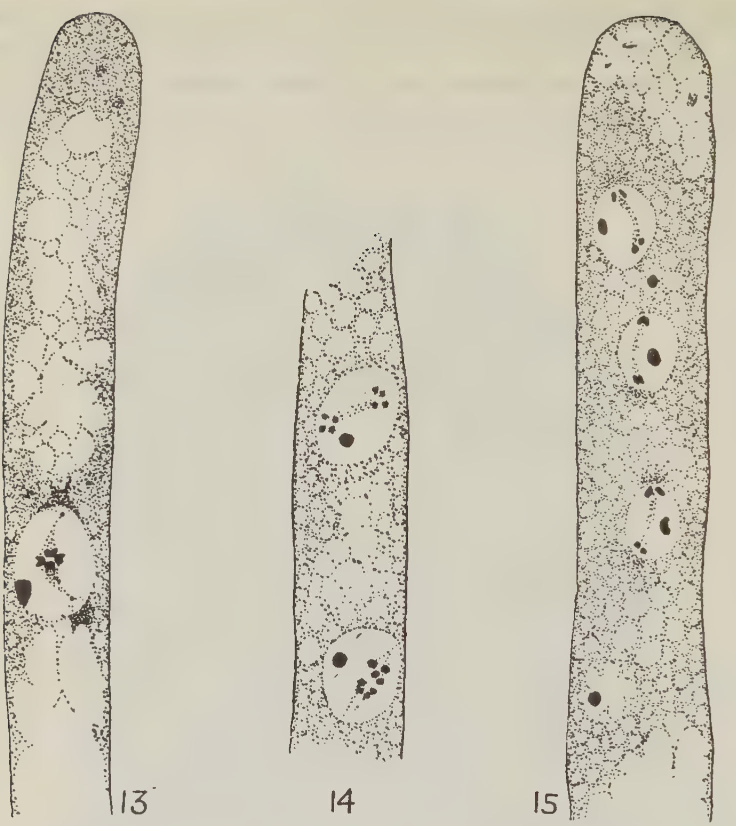


FIG. 12. An ascocarp in longitudinal section, older than that in Fig. 10; the paraphyses are beginning to separate and are overtopped by the cells of the outer sheath, one cell of the coil is forming ascogenous hyphae, the cells of the trichogyne are nearly empty. × 700.

Only a cursory study has been made of the nuclei. Division in the nucleus of a spore indicates the presence of two chromosomes, the haploid number; in the divisions in the oogonial region and aseptate ascogenous hyphae the number appears to be four. Four gemini are recognizable on the first



FIGS. 13–15. Nuclear divisions in asci. × 2,600. Fig. 13. Metaphase of first meiotic division with four gemini. Fig. 14. Anaphase of second meiotic division. Fig. 15. Telophase of third division in ascus.

meiotic spindle in the ascus (Fig. 13), four chromosomes throughout the second division (Fig. 14), and two (Fig. 15) in the telophase of the third.

SUMMARY

1. *Lachnea melaloma* is heterothallic; binucleate spores and self-compatible mycelia are occasionally found. Oidia are formed in single-spore culture.

2. Aoscopores germinate readily at 20 to 25° C.; a variety was collected in which the spores required preliminary heating.

3. The primordium of the ascocarp is a coiled branch extended distally as a trichogyne-like outgrowth. This trichogyne does not function, but fusions are common between vegetative hyphae and the stalk of the coil or the branches from its proximal cells.

4. The middle cells of the coil form an oogonial region, two or more cells being in continuity by means of wide protoplasmic strands. Ascogenous

hyphae arise from one, two, or possibly more of these cells. Nuclear fusions in the oogonial region take place singly, indicating that normal fertilization does not occur.

5. The first division of the definitive nucleus of the ascus shows four gemini, four chromosomes are visible throughout the second division, and two in the telophase of the third. There is evidence that the nuclei of the vegetative cells contain two chromosomes and those of the ascogenous hyphae four, the diploid number.

6. No indication was found of the formation of a male branch; the trichogyne appears to be vestigial, and compatible nuclei to be associated by means of mycelial fusions.

Thanks are due to the authorities of the British Museum (Natural History) for kindly naming the fungus.

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The Development of the Vascular System in Evergreen Leaves More than One Year Old

BY

J. H. ELLIOTT

(*Department of Botany, University of Leeds*)

With seventeen Figures in the Text

IN an earlier study of leaf development (Elliott, 1933) it was shown that xylem production is complete throughout leaf and trace by the time that leaf expansion ceases, although phloem production may continue for some time. So far consideration had been restricted to the leaf behaviour during the first growing season, but the early cessation of xylem production naturally raised the question as to the behaviour of evergreen leaves in the second and later growing seasons. This question has therefore been examined with results that will be summarized in the following pages. Attention was paid mainly to plants growing in the open, so that the choice of hardwood species was necessarily limited and mainly restricted to shrubs and bushes—most of these not being indigenous. Amongst softwoods it was, of course, possible to examine the leaves of many well-developed tree types.

The persistence of the leaves varies in different species, and while many shed their leaves during the third season, e.g. Escallonia, Pyracantha, &c., others retain them for as long as five years (Ficus, Rhododendron), but in these cases the plants are usually growing in sheltered conditions.

The vascular development of evergreen plants has hitherto been neglected, and examination of the literature reveals that attention paid to these species has been divided between their structure in relation to water loss and the ecological distribution of the evergreen flora.

Detailed studies have been made by Guttenberg and Bergen (1903) on the Mediterranean coast flora, by Cooper (1922) on the broad-leaf sclerophyll vegetation of California, and by Cannon on desert plants in general. Thoday (1931), Delf (1915), and others have discussed the structure of many evergreen leaves in relation to transpiration, but in all the papers it is evident that the actual anatomical relations of stem and leaf vascular systems have been ignored. Farmer (1918), in investigating the conductivity of evergreen woods, connects the slow growth of the trees with the low conductivity of the wood which can transmit only a limited amount of water. This certainly has more bearing on the problem, and a direct reference is made by Frank (1864), who records an increase in vascular tissue in two-year-old Holly leaves. Again, in

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the conifers, Frank recorded small additions to phloem and xylem in leaves more than one year old, but there is little evidence as to the actual development of the vascular tissues. Tison (1902–21*a*) has described the additions to the trace xylem in the stem wood region in successive years in various species, showing how the older elements were ruptured while new tracheides were added on the under side. Similar observations have been made upon Araucaria by Seward (1906) and Markefeldt (1885), whilst these and other workers (Thiselton-Dyer, 1901) have recorded the unique circumstance which occurs in this group, namely, the renewal of the traces in the stem for many years after the leaf has fallen.

Tison (1902–21*b*) has also described the steps which occur in the final breaking of the trace in the conifer and the dicotyledon leaves at leaf-fall, indicating how the break occurs in the cambial region and how the cambium becomes continuous over the broken end of the trace in succeeding layers of wood.

In general, however, the whole question of vascular development in the evergreen leaf has received but little attention, and it is hoped that the following observations will throw some light on the general problem.

General Description of the Vascular Tissues in the Adult Evergreen Leaf

It will be advisable first to describe the general structure of the vascular bundle of the adult first-year leaf.

In the greater number of the species examined the vascular bundle is a crescent-shaped structure with the pointed ends turned inwards and the concave face towards the upper surface of the leaf. The bundle is always placed centrally in the petiole and main vein, but in some species—Ficus, Hedera, &c.—there are several small bundles instead of one large bundle. The xylem is always on the upper side of the bundle and is of distinctive appearance. In the greater number of cases the xylem elements are separated into groups of 1–3 files of vessels by unlignified rays, so that there is not a complete lignified band of xylem tissue as in many of the deciduous species. The separation into files is particularly evident in the region of the narrow metaxylem elements. In Rhododendron, and in the allied genera Azalea and Arbutus, the rays usually become lignified so that the wood does have the deciduous-type appearance. In actual character the xylem elements are quite normal, except that large vessels are rarely found (with the exception again of Rhododendron and allied genera which do have fairly large vessels in the spring wood). The xylem develops the usual type of thickening, but there is a greater tendency than in the deciduous types to form scalariform thickenings. Except in Rhododendron and Arbutus, fibres and tracheides are not common.

The phloem is also present in groups of two or three files of cells—the ray cells separating the files are usually very large. The actual phloem cells of the evergreens are rather small, a description which applies particularly to the sieve-tubes. These elements are almost always wider tangentially than radially, so that it is evident that they undergo but little expansion. In most cases they are of the normal type with one companion cell, but in *Pieris floribunda* Bth. and in Laurustinus the later formed and narrower tubes have been observed to possess two such cells. The phloem cells of evergreens are inclined usually to have rather thick walls, even in the sieve-tubes, whilst the plates of the latter have very small perforations. The companion cells are of the normal type, but are rather larger in proportion to the size of the sieve-tube than is the case in the phloem of the deciduous leaves.

THE FURTHER DEVELOPMENT OF THE VASCULAR SYSTEM IN THE LEAF ITSELF IN EVERGREEN DICOTYLEDONS AND CONIFERS

In the deciduous leaf xylem formation ceased when leaf expansion ceased. It is important to know, therefore, whether any further increase in size of evergreen leaves takes place after the first growing season. Superficial observations on this point are rather misleading, as adult leaf size will vary somewhat according to the season, and the crop may be of greater average size one year than for several previous seasons.

The matter has been investigated fairly thoroughly in *Rhododendron Ponticum* L., *Prunus Lauro-cerasus* L., and *Ilex Aquifolium* L. Leaves of these three species were measured at the end of the first growing season and the same leaves were measured again during the second and third years (when not lost by accident, &c.). In no case has any increase in size been recorded at any period subsequent to the cessation of growth at the end of the first growing season, either in length or width of the leaves. In the remaining species examination has not been so thorough, but numbers of leaves have been measured (from the same plant in many cases), and it has always been found that the *average* length of the first-, second-, and third-year leaves is the same. Particularly is this the case in regard to evergreen leaves from plants grown indoors where the surrounding conditions remain fairly constant. In Hedera some increase in petiole length was occasionally recorded, although in the majority of instances this plant behaved like the others. There is a possible explanation of this case which will be advanced when the following observations are discussed.

These observations relate to a slightly different type of growth in which second-year increase in leaf size has been observed to some small extent. This is the case of the 'lammas growth' leaves of *Rhododendron Ponticum* L., a species which is much subject to this late growth. The newly formed buds begin to grow out in August and September, but growth usually stops when the leaves have reached, at most, one-third of their normal size, and in many cases when they are much smaller than that. During the winter they undergo a resting period, but frequently increase in size a little during the following spring. This second growth period lasts only for a short time and the leaves never approach normal size.

It is quite possible that this second growth period is merely a delayed continuation of the unfinished autumn growth and cannot be cited as an example of a second year recommencement of growth in a normal adult leaf. It may also be that in Hedera there is a parallel case, except that here it is the normal growth that is not completed during the first year and the increase in petiolar length which has been recorded is but a continuation of the first-year growth. (Hedera does not often show definite lammas growth.)

Lammas growth has also been observed occasionally in *Prunus Lauro-cerasus* L., but it starts rather earlier and is completed the same autumn, no cases of renewed growth having been recorded. Also in this species lammas growth is more vigorous, and the leaves produced at this time usually attain the full normal size for the species.

Lammas growth has not been observed to any notable extent in the other evergreen species.

Vascular Development after the First Growing Season

The leaves of all the species mentioned have been carefully examined in the second season in order to detect the appearance of new xylem and phloem. The recognition of newly formed xylem is much more easy than the detection of new phloem, as when the xylem appears it takes the form of a band of larger vessels following the smaller summer wood elements of the previous year. The new xylem also frequently stains much more deeply than the neighbouring summer wood. No such criteria are available for the recognition of new phloem.

Of the species examined up to the present time, new xylem has only been seen in the second-year leaves of Rhododendron, *Prunus Lauro-cerasus* L. and *Arbutus Unedo* L. These cases will be dealt with separately and in detail.

Arbutus Unedo L. (Figs. 1 and 2).

Owing to the difficulty of obtaining specimens of this plant it must be stated at once that observations have been necessarily confined to two or three shrubs. On these plants there were no three-year-old leaves, but in all the two-year-old leaves examined on one plant there was a definite band of second-year xylem—more in some leaves than in others. In the other plants the appearance of second-year xylem was frequent, though much less regular, and while some leaves showed quite a wide zone, others had only a narrow band. The leaves of this species always show very much more of this second-year xylem than either of the remaining species. The new xylem consists of a zone of wide spring vessels and then a zone of narrower summer ones, among which are occasional wide ones. The amount of summer wood formed is much less than in the first growing season. The second-year xylem is present up into the main vein but decreases in amount, particularly in the quantity of summer wood. An important feature of this leaf is that where there is a vigorous production of second-year wood there is also an increase, though a much smaller one, in the amount of xylem in the pulvinus, the additional xylem in the latter region consisting of narrow protoxylem vessels. In leaves with but little second-year wood only a few new elements appear in the pulvinus. This is the only species examined in which second-year xylem appears in quantity in the pulvinus, but even in this case it is not continuous into the trace. In all cases of second- year xylem formation in the leaves its inception is after the recommencement of cambial activity in the stem under the influence of the growth of the buds above.

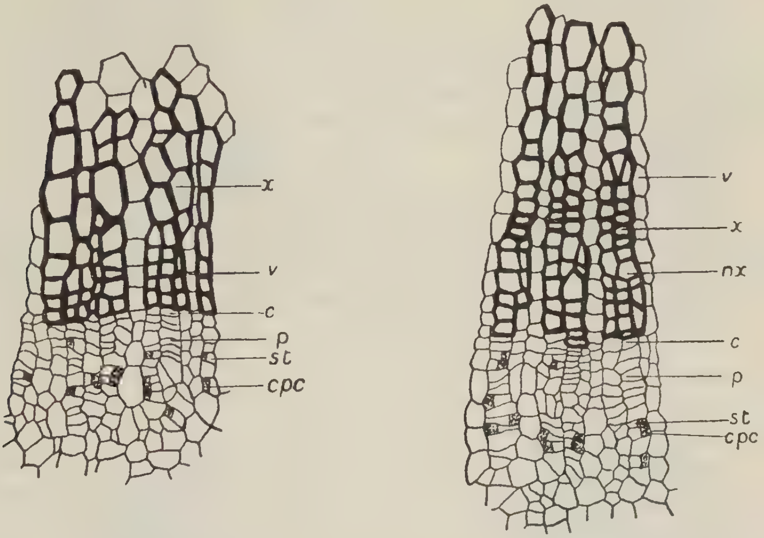

FIG. 1. FIG. 2.

FIGS. 1 and 2. *Arbutus Unedo* L. Fig. 1. First-year leaf. xylem, *x*; phloem, *p*; sieve-tube, *s.t.*; companion cell, *cp.c.*; cambium, *c*; ray, *r*. (The same lettering is used in all figures from 1 to 12.) Fig. 2. Second-year leaf. A new ring of xylem several cells deep is present, but there has been no appreciable addition to the phloem (new xylem, *n.x.*).

Rhododendron Ponticum L. (Figs. 3, 4, and 5).

The production of second-year xylem in this species is frequent but irregular and of small quantity. In many cases it is restricted to certain faces of the old wood, so that in such instances there is not a complete band of new xylem.

The new xylem consists of fairly large vessels with odd smaller elements, and is definitely cut off from the leaf cambium in the second year. It does not appear until after cambial activity has started in the twig and the new buds are growing. This point has received special attention because of the evidence it provides with regard to the connexion of the old leaves with the recommencement of cambial activity in the stem. Xylem production in the leaf soon ceases again and no summer wood is formed, so that cambial activity is again brought to a standstill. The production of second-year xylem does not seem to be restricted to any particular type of leaf, and while it has always been found in leaves bearing a branch in the axil—usually vigorous leaves—any leaf which is actually healthy seems to be capable of forming second-year xylem.

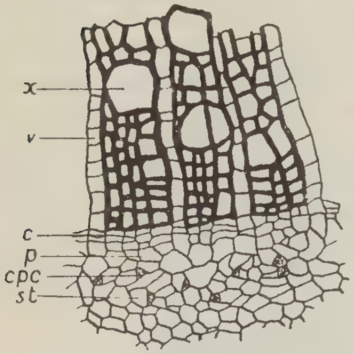

FIG. 3.

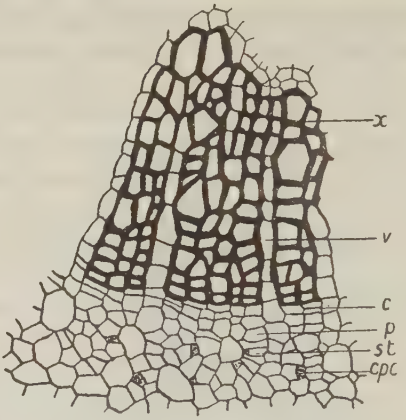

FIG. 4.

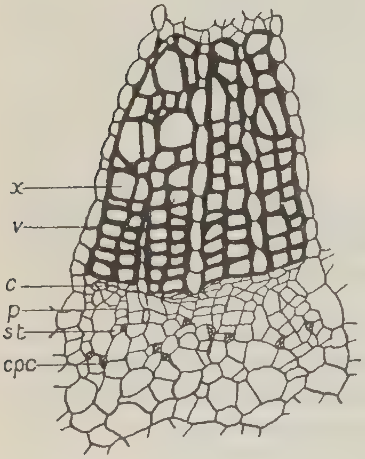

FIG. 5.

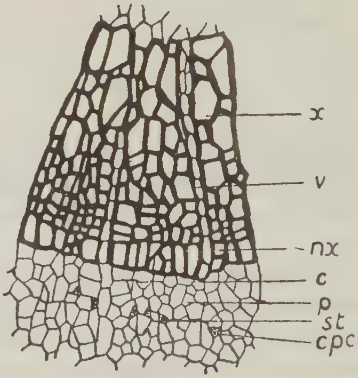

FIG. 6.

FIGS. 3–6. *Rhododendron Ponticum* L. Fig. 3. First-year leaf. Appearance of xylem and phloem. The cambium cells are still tangentially flattened. Fig. 4. Second-year leaf. Appearance of xylem and phloem. There has been no increase in either xylem or phloem, but the cells of the phloem are more expanded. The cells of the cambium have vacuolated and expanded. Fig. 5. Five-year-old leaf. No more xylem or phloem has been added and the cambium has collapsed. Fig. 6. Second-year 'lammas growth' leaf. A new ring of xylem has been added, but no more phloem. The cambium has vacuolated (new xylem, *n.x.*).

The above statements apply to the normal crop of leaves. The 'lammas growth' leaves always have a band of xylem cut off in the spring of the year following the first growth, and this xylem also is cut off by fresh divisions of the leaf cambium following the commencement of cambial activity in the stem. No summer wood is produced, and the renewed activity of the leaves lasts only for a short time and does not occur in succeeding years (Fig. 6).

As in the case of the increase in leaf size, it is felt that this new formation of xylem is to be regarded as the delayed continuation of the normal growth and not as a recommencement of growth, particularly as it never occurs in succeeding years. If this is so, then the conditions occurring in these leaves do not affect the general considerations.

Cherry Laurel (*Prunus Lauro-cerasus* L.) (Figs. 7 and 8).

The production of second-year xylem in this species is very occasional and is never of great extent. Such production is usually confined to odd vessels, although occasionally a very narrow but complete band of new tissue is produced. This is usually found in large vigorous leaves with a branch in the axil. Any new tissue produced appears after the commencement of cambial activity in the stem. Very little, if any, new xylem is formed in the 'lammas growth' leaves in the spring succeeding their first growth, as in almost every case they are fully formed before growth stops in the autumn.

An experiment has been performed with Cherry Laurel, but not yet completed in other species. Several twigs were completely disbudded, during March, down to the four-year-old stem, i.e. below any leaves on the stem. At the end of the year the twigs were removed and the various regions were preserved separately. Many of the older leaves were shed, much more so than on the uninjured twigs.

None of the leaves from these shoots showed any signs of second-year xylem production, and the leaf cambium was not recognizable as a meristematic tissue (Fig. 9). Moreover, there was no further cambial activity in the stem, except in one case where a bud had been overlooked for some time. Below this bud there was a crescentic band of new xylem. Lignification of the new xylem was very incomplete. Elsewhere no new tissue had been formed.

The object of this experiment was to determine the influence of the old leaves on the recommencement of cambial activity in the plant. The results show quite plainly that in this case they are incapable of starting any new growth, and further that they cannot participate in additional vascular activity unless stimulated by the growth of the buds.

The evergreen dicotyledons in the following list have also been examined, and in these no second-year xylem has been found in the leaves at all above the pulvinus.

Andromeda sp.
Aucuba japonica Thunb.
Azalea sp.
Buxus sempervirens L.
Camellia japonica L.
Eleagnus pungens Thunb.
Escallonia floribunda Kunth.
Euonymus japonicus Thunb.

Ficus repens Hort.
Griselinia littoralis Raoul.
Hedera Helix L.
Hypericum Androsaemum L.
Ilex Aquifolium L.
Myrtus communis D.C.
Olearia Haastii Hook. f.
Pieris floribunda Bth.
Pyracantha coccinea Roem.
Veronica Andersonii Ldl.
Viburnum Tinus L.

Counting of the vessel files in leaves of different ages and the use of the other criteria mentioned has failed to show any increase in the amount of xylem after the first year. Comparisons of the size of the vein islets in leaves of different years has been made, but there is nothing to show that new veinlets have been formed or that renewed growth of the existing ones has occurred.

In the pulvinus vessels are produced occasionally and very irregularly during the second year, but comparison of numerous leaves indicates that they do not appear after the second year even when the leaves live for five years. Such statements have to depend on comparison of numerous leaves because an individual leaf can only be examined once.

THE CAMBIUM

During the active growing period of the leaves the general appearance of the cambium is one layer or two layers of rather thin-walled cells. In these slow-growing plants there is never a very wide region of dividing cells, neither are these cells compressed in the manner usually seen in the dicotyledon trees. They have thin tangential walls and are almost as wide radially as tangentially. After the first growing season it is usual to find that the cells lose their meristematic appearance. They expand a little and frequently vacuolate, so that they look more like a regular row of parenchyma cells.

In *Griselinia littoralis* Raoul (Figs. 10, 11) radial divisions occur in the old cambium, so that the cells are no longer in radial series with the adjoining xylem elements, while in Laurustinus the cells frequently elongate in the radial direction so that the cambial appearance is finally lost. The extreme case of cambial differentiation has been seen in Andromeda, where, in one or two cases, the cambium cell (or at any rate the cell next to the xylem) had become a sieve-tube and cut off a companion cell. Such a condition has not so far been found in other species.

On the other hand, particularly in *Prunus Lauro-cerasus*, the cambium is often so completely crushed that it is almost impossible to recognize individual cells.

In almost every species differentiation of the cambium is more prominent and more advanced in the petiole than in other parts of the leaf.

From the above observations it would seem that the evidence points in the majority of evergreen species to a cambial layer incapable of division to form new vascular tissue after the first season.

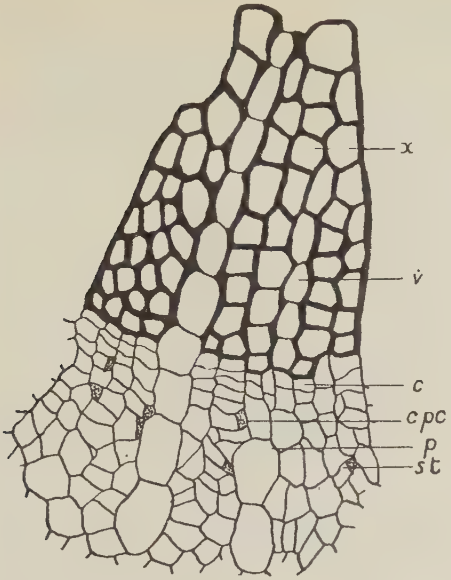

FIG. 7.

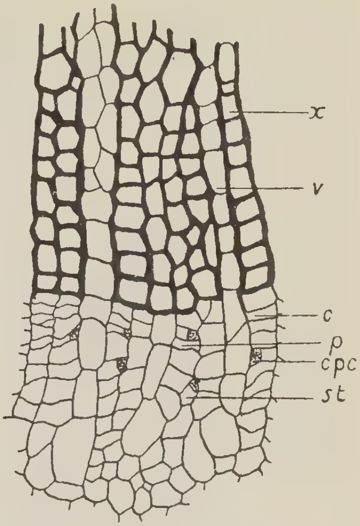

FIG. 8.

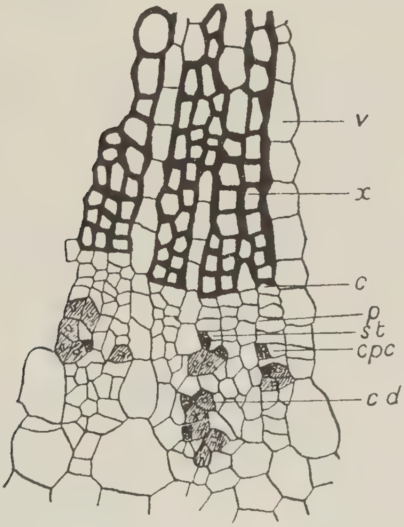

FIG. 9.

FIGS. 7–9. *Prunus Lauro-cerasus* L. Fig. 7. First-year leaf. Arrangement of xylem and phloem. Fig. 8. Second-year leaf. No more xylem or phloem has been added, but the cambium is still recognizable. Fig. 9. Second-year leaf from disbudded twig. No more vascular tissue has been added, the cambium has vacuolated, all the phloem walls are abnormally thick and cellulose deposits (*c.d.*) almost block some of the cells.

THE PHLOEM

Examination of this tissue provides the most unexpected results in the whole investigation. In none of the species so far examined can it be said that any phloem was produced after the first year of growth. Even in Arbutus, which showed such a definite band of second-year xylem, it is very doubtful whether any new phloem was present. Admittedly the appearance of new phloem is difficult to detect in small quantities, but examination of leaves at the end of the first growing season and during the succeeding seasons, and comparisons of first-, second-, and third-year leaves at the end of each year, have failed to reveal any increase in the numbers of phloem cells visible in transverse sections of various parts of the leaf.

Occasionally a little of the first-year phloem is not differentiated at the end of the year, but usually the sieve-tubes are then quite empty. In the succeeding seasons the sieve-tubes are not crushed and the companion cells frequently vacuolate and expand a little. In many cases, e.g. Laurustinus, *Prunus Lauro-cerasus*, Pieris, and others, these cells do not expand but become filled with a densely staining material. Usually all walls in the phloem region become heavily thickened with cellulose. Fatty or lignin impregnation has not been recorded, and there is no indication that the thickening is of a gummy or mucilaginous nature. Thickening of the phloem walls frequently occurs before the end of the first year, and in the later years may increase to such an extent that in *Prunus Lauro-cerasus* and Laurustinus the lumen of the sieve-tube is almost completely obliterated. This condition was particularly marked in the leaves of the disbudded Prunus twigs. (It may be noted that, even when the deciduous leaf is about to fall off the tree, there is no thickening of the phloem walls.)

The general condition of the phloem in old evergreen leaves suggests that it is utterly non-functional, and in section it is often difficult to obtain a clear cell outline, as though the walls, in spite of their thickness, were soft and easily crushed. Under such conditions it is difficult to suppose that any translocation can occur.

CONIFERS

The following conifer species have been examined and show some differences in the condition of the leaf vascular system after the first year:

Araucaria imbricata Pav.
Cedrus Libani Barrelier.
Cryptomeria japonica Don.
Picea excelsa Link.
Pinus sylvestris L.
Taxus baccata L.
„ „ *v. fastigiata* Loudon.

In these leaves there is no petiole. A single vein, which may be composed of two bundles lying side by side, traverses the leaf in the needle-leaved species, although in Araucaria there are many convergent veins. In no case are there any veinlets, and the single vein does not branch. In some species there is an endodermis surrounding the bundle. The amount of vascular tissue in these leaves is very small and the number of elements seen in transverse section can be counted quite easily. Besides the normal xylem elements, but outside the bundle, the so-called transfusion tracheides occur in groups in many species.

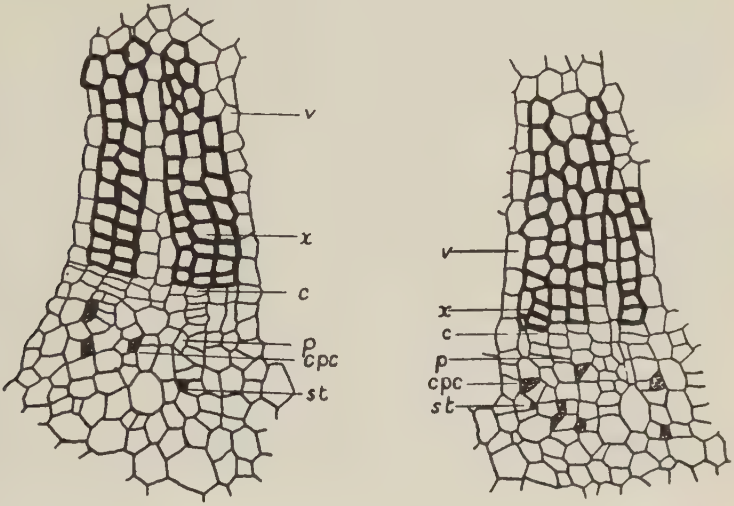

FIG. 10. FIG. 11.

FIGS. 10 and 11. *Griselinia littoralis* Raoul. Fig. 10. First-year leaf. Arrangement of xylem and phloem. Fig. 11. Second-year leaf. No more xylem or phloem has been added and the cambium has vacuolated and is no longer meristematic.

Growth in size of the leaves appears to be completed during the first year, but there seems to be a greater tendency for variation in leaf size from one year to the next.

Counting the number of xylem elements in the various species from year to year shows that there is no appreciable production of new xylem after the first year, or rather after the leaf has reached adult size, because it is difficult in some cases to say whether the leaf is fully grown at the end of the first year. If any extra xylem does appear it is produced as an additional file at the edge of the existing tissue and not by addition to the files already present. The amount so formed, however, is quite negligible, and no conifer so far examined is comparable with Arbutus.

In the phloem there is a definite increase in the amount of tissue present up to and including the third year, and at the end of this period also there is the maximum amount of *active*, uncrushed phloem. After the third year no more tissue is produced, but collapse of the existing cells continues, so that ultimately there is only a very narrow zone of non-collapsed and presumably active phloem. The walls of the phloem cells are never thickened before they are crushed, and they then appear thick because they become just a shapeless mass in which individual walls or cells are unrecognizable (Fig. 12).

The cambium also is never thickened or vacuolated, and at all times remains meristematic in appearance.

The species *Araucaria imbricata* Pav. must be mentioned separately. This plant retains its leaves alive for over thirty years, during the first few of which they increase in size by normal growth, while later on they become broader in a manner to be described. After the first year or two there is no increase in the amount of xylem in the original bundles, but it seems certain that new ones arise. An interesting feature is that the 'transfusion-tracheide' tissue, though not included in the vascular bundle, increases from year to year and is present in considerable quantities in the oldest leaves.

As the radial dimensions of the shoot increase the leaf base is stretched, and this appears to be accommodated by the proliferation of the mesophyll, although this stretching also results in the splitting of the epidermis and the formation of cork beneath that layer. As the stem gets older the basal portion of the leaf is buried in, and forms part of, the stem cortex, so that the true veins of that part of the lamina become part of the leaf trace (Figs. 13, 14, 15). The leaf becomes much thicker all over, but the distal part does not become broader, a fact which suggests that the increased width of the base is not due to an organized growth such as was responsible for the first expansion of the leaf.

In the conifers as a whole one finds that there is a contrast to the evergreen dicotyledons in the production of new phloem for a few years, but consideration of both groups of evergreens points definitely to the conclusion that cambial activity in leaves more than one year old continues, if at all, at a very much reduced rate, and there is no vascular activity which is comparable with that of the first growing season.

THE RELATION OF THE LEAF TRACE WITH THE STEM VASCULAR SYSTEM

During the first year of leaf growth, whether deciduous or evergreen, conifer or angiosperm, the vascular system of the leaf is developing in direct continuity with that of the stem (Fig. 16). In the extending stem all the xylem elements are continuous through the leaf traces into the extending leaves. All this xylem is protoxylem, but is continuous with the new metaxylem produced on the face of the old wood of previous years. Later in the summer, when extension has ceased, a new zone of wood is produced in the young twig, and this is not in continuity with any wood in the now adult leaf. The important feature which emerges from this introduction is that, in the angiosperms particularly, at all times during the first year the leaf xylem is continuous with the stem xylem only through protoxylem—under no conditions has metaxylem been found in the leaf trace of these plants. At this stage the deciduous leaf is shed and passes out of consideration.

What changes, however, take place in the leaf trace of the evergreen in association with the persistence of the leaf? It has been pointed out that only in a few species, and then not regularly, has a new zone of vascular tissue been produced in the evergreen dicotyledon leaf after the first year and that this is never continued down into the trace. In the latter, therefore, one finds only the protoxylem produced during leaf extension. During the succeeding years new bands of xylem are formed annually in the twig under the influence of the elongating shoots above, and so the radial dimensions of the twig are increased. As the stem is no longer elongating, all this xylem is metaxylem. The major point which is to be made at present is that in no evergreen dicotyledon so far examined has any leaf trace been found in which additional xylem elements have been produced to make continuity with the present year's wood. This point has been extremely carefully investigated and the traces examined in all possible views to elucidate it, but so far it has been impossible to establish connexion between the trace protoxylem and the new wood in the stem. This means that the leaf, although still attached to the stem, has no direct continuity with the new xylem formed in that stem, or, in other words, the leaves produced in any particular year are isolated from the new growth of the succeeding years.

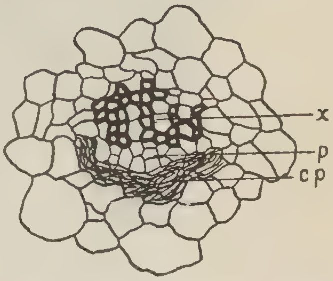

FIG. 12. *Cryptomeria japonica* Don. The vascular system in the leaf (collapsed phloem, *c.p.*).

In all the dicotyledon evergreens examined there is a very wide leaf gap and the leaf-trace xylem is surrounded on all sides by parenchyma, so that there is no part of the trace in contact with any of the stem xylem in its neighbourhood. This means, that although new wood is produced by the stem cambium on either side of the leaf trace and below it, this new wood is quite distinct from the trace. In tangential section the stem wood elements can be seen bending around the trace and forming a complete zone again lower down, but none are observed to enter the trace.

Considerable strain must be exerted on the trace as the stem gets thicker, but this is doubtless minimized by the sharp angle at which the latter crosses the stem wood, and also by the fact that the size of the leaf gap prevents pressure being exerted vertically upon the trace by the mass of stem wood above—a factor which comes into play in the conifers.

Thus in the evergreen dicotyledons there is the condition that the xylem of the leaf trace is always protoxylem, though it is in continuity with metaxylem lower down in the old stem, and that under no conditions is the continuity of the leaf trace with the stem wood renewed from year to year, an observation which can also be extended to the phloem. So there is complete isolation of the leaf from the stem vascular tissue produced after the leaf has become adult.

In the conifer there is quite a different sequence of events. In all the species examined the leaf trace, in the stem cortex, contains metaxylem even before the end of the first year. While the young leaves and shoot are growing there is protoxylem only, but when the last-formed metaxylem elements of the leaf are developed they are continuous with the summer wood of the stem through metaxylem tracheides in the leaf trace. These tracheides are both pitted and scalariform.

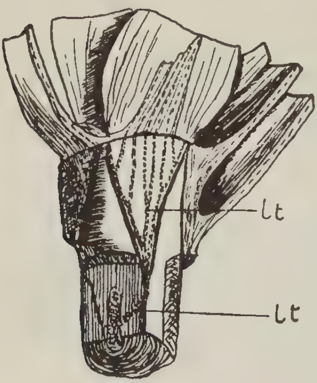

FIG. 13.

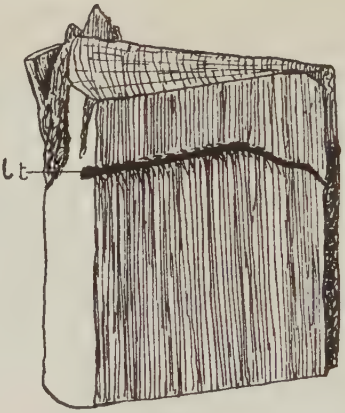

FIG. 14.

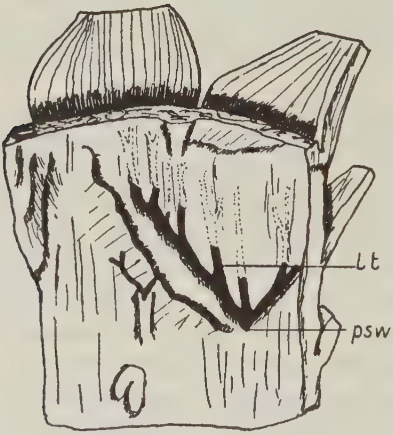

FIG. 15.

FIGS. 13–15. *Araucaria imbricata* Pav. Fig. 13. Dissection of part of a six-year-old twig to show the branching of the leaf trace in the cortex and the path of the trace across the stem wood (leaf trace, *l.t.*). Fig. 14. A section of an old stem (approximately 30 years) showing the course of the persistent leaf trace across the stem wood. The leaves were still living on this stem. Fig. 15. Dissection of the bark of an old stem (30 years) to show the further branching of the leaf trace system (leaf trace, *l.t.*; point of trace entry into stem wood, *p.s.w.*).

In those conifers in which leaves are borne directly on the main stem and not on short shoots, e.g. Picea, Araucaria, Cryptomeria, &c., these leaves possess long and decurrent leaf bases. In all such cases the course of the leaf trace is a very steep one through the stem cortex, so that actually the trace enters the stem vascular system at about the point at which the decurrent leaf base ends on the outside of the stem. Thus there is a long portion of the trace in the stem cortex, a condition which is not found in the dicotyledon types. Where the leaves are borne on short shoots, e.g. Pinus, Cedrus, the leaf trace appears to pass straight across the cortex into the stem vascular system. It may be noted that as a general rule these short-shoot leaves do not persist for many years.

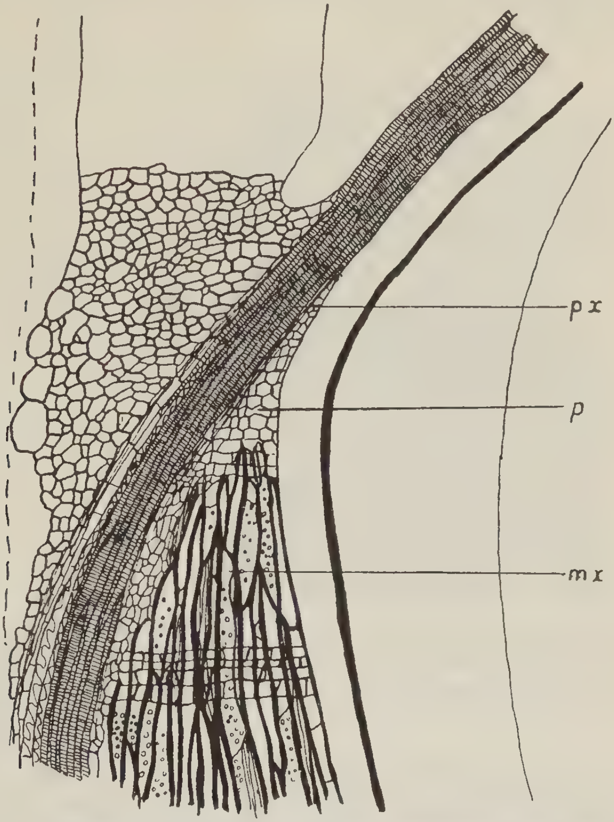

FIG. 16. *Pieris floribunda* Bth. Junction of the leaf trace with the stem wood. The protoxylem (*p.x.*) of the trace is obviously not in continuity with the younger metaxylem (*m.x.*) in the stem. A block of parenchyma (*p.*) appears below the trace level with the leaf gap.

The conifer trace enters the stem vascular cylinder in the first type of shoot at a very acute angle, but very early shows a concavity on the upper (inner) surface—that is to say, it presents a step-like appearance. It seems extremely probable that this condition is due to the pressure of the stem wood on the trace in this region causing it to be bent more and more as the stem wood gets thicker. The pressure of the stem wood is a result of the small leaf-gap, and soon causes a snapping of the older elements of the trace. When the latter is at last bent at right angles to the stem wood it becomes subject to further strain as the radial dimensions of the stem increase and so more trace elements are broken.

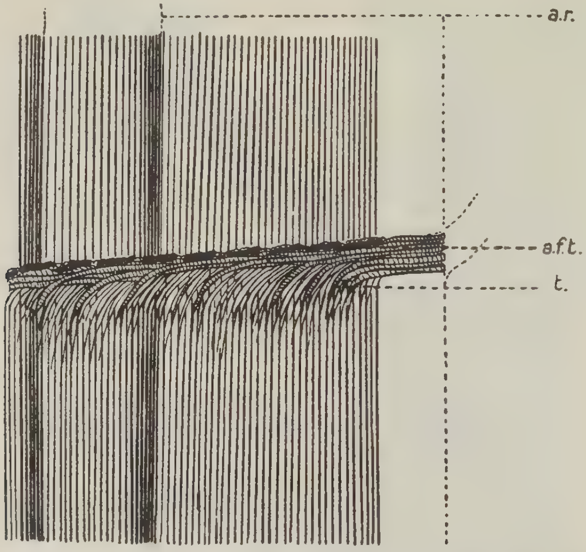

FIG. 17. *Cryptomeria japonica* Don. The junction of the leaf trace with the stem wood (semi-diagrammatic). The succession of tracheides which maintain continuity between trace and stem wood are clearly shown (*t.*) on the under surface of the trace. On the upper surface (blocked in) are the broken air-filled tracheides (*a.f.t.*) (*a.r.* end of annual ring).

In contrast with the dicotyledons, however, it is found that in all cases the connexion between trace and stem is renewed each year, even up to and beyond the thirty-year stage observed in Araucaria. A radial section of the wood in the stem of any of these species, cut along the leaf trace, shows a horizontal trace from which tracheides can be seen curving down into the stem wood below the trace. There is no doubt whatever that these elements belong to the trace. On the upper face of the latter there is a narrow parenchyma layer, and just below this can be seen the broken air-filled tracheides which made the connexion in an earlier year and which have been broken by the increase in stem diameter (Fig. 17).

There is no sudden snapping of the leaf trace to leave a gap, but the oldest tracheides, which have undergone most stretching, break first, while new tracheides are cut off on the under side of the trace by the trace cambium. Thus the trace gets very little thicker in the wood because the older portion is always left behind, the newer pieces being inserted, as it were, between that part of the trace xylem already in the wood and that part which is in the stem cortex. So it follows that the trace is never discontinuous. The effect of such growth is evident in the trace xylem in the cortex, because in this region there is no breaking and the trace becomes thicker—though very slowly because here new tracheides are added only infrequently. This addition of tracheides becomes even more rare as the trace approaches the leaf base, and by the time the trace enters the leaf all addition of vascular tissue has ceased and the bundle is in the non-active condition already described for the leaf. Such a condition is particularly obvious in the oldest leaves of Araucaria where, when the phloem and cortex are removed from the stem, a stout peg of trace xylem can be seen protruding from the stem wood. If the trace is dissected out from the cortex it is observed to become increasingly narrow and a section of the upper part shows the leaf condition (due to the fact that as the leaf gets older, the basal part of the leaf, and with it the veins, is buried in the cortex).

It seems certain that in all cases the curved tracheides are cut off by the 'armpit' cambium extending from the main sheet of cambium up the incoming trace. The character of the additional xylem cut off in this manner each year is undoubted, it is all metaxylem, though not always of the same type. In Taxus and Cryptomeria the new tracheides have rather close scalariform thickenings, that is to say, the areas of primary wall are very narrow and laterally elongated. In Picea similar thickenings are also observed, but there are in addition bordered pits, whilst in Araucaria all the new elements seem to have bordered pits as well as scalariform thickenings. There is no doubt that when young these scalariform tracheides could undergo a certain amount of stretching, but this cannot go on for long, and when older they are broken. The broken tracheides become filled with air and are easily seen.

The phloem condition is not so easy to follow. Certainly in the species mentioned there is an increase in the amount of phloem present in the trace up to the third year, but after this it is difficult to estimate, and certainly no more appears in the leaf. In the stem cylinder there is an annual increment of phloem, but it is difficult to say whether any of this is produced from the trace cambium, and at the same time there is no means of distinguishing between stem and trace phloem as there is between stem and trace xylem.

Special consideration must again be given to the case of Araucaria, being the only species in which the leaf trace is renewed year after year when the leaves have fallen off. The continued increase is only maintained in that part of the trace which crosses the stem wood, and also in a short peg of the trace protruding from the stem wood. Thus the point of entry of the old leaf trace into the stem wood is always visible when the phloem and cortex are stripped off. When, if ever, the additions to the trace xylem cease to develop has not been ascertained, but it is certain that the renewal is continued up to thirty years after the leaf has been shed. Such a proceeding necessarily presents a serious obstacle to any suggestion that the continued production of elements into the trace xylem is due to an influence exerted by the leaf.

DISCUSSION

The facts put forward in the preceding pages make it obvious that a sharp distinction must be drawn between the behaviour of the evergreen dicotyledons and the conifers.

In the dicotyledons it has been shown that xylem production in leaves more than one year old is definitely the exception, and in fact only three cases could be cited. Even in those instances there is no addition to the trace xylem in the region of the stem wood, so that the growth of the latter would appear to be unaffected by the changes in the leaf vascular system.

No species has been found after the first year to show an increase in the amount of either leaf or trace phloem, so that one would expect that translocation would be very much reduced. Such a suggestion is based on the idea that the greater part of the translocation through the phloem is during differentiation, as suggested elsewhere (Priestley, 1930), and also on the fact that many of the old sieve-tubes are blocked by cellulose deposits. If translocation is so much reduced, it means that the old leaves of an evergreen can be making little contribution to the plant as a whole in the way of photosynthetic products.

On the other hand, the water-conducting systems of the leaves of these plants are not in direct continuity with the new wood produced each year, and so from these facts it would appear that the second-year and older leaves of an evergreen cannot be regarded as an active factor in influencing the growth of the plant. Unfortunately few data are available on the photosynthetic activity of second-year leaves. Are we to regard them as merely making sufficient food to balance their own respiration, or is there an accumulation of carbohydrate which cannot be translocated? There would seem to be some evidence of the latter condition in the thickenings of the phloem walls which have been recorded in these species, and again in the deposits of cellulose.

On the present evidence the writer is convinced that normally the leaves of evergreen dicotyledons, even though they remain on the tree for five years, do not influence the radial activity of the stem after the first year. Such a lateral organ as the leaf, having no direct continuity with a newly growing region above, is not stimulated seasonally to further activity as is the stem upon which it is borne. If and when there is a further period of activity in the leaf vascular system it is not due to a seasonal impulse but to an unusual combination of factors which render further cambial divisions possible.

In the conifers there is a different state of affairs. Certainly in most cases there is no further xylem production, but up to the third year new phloem is formed in the leaf. How can this be interpreted on the lines suggested above? It has been shown quite clearly that the continuity of the trace is renewed from year to year. Does this mean that under the influx of water thus ensured the cambium keeps active in the production of phloem for a short period, so that up to the third year there is an increasing zone of active phloem? If this is so then one is driven to the conclusion that even though the trace is renewed each year the increasing number of young leaves above any particular section of the twig considered gradually diverts the water supply from that part, and later activity again falls off, with the result that the phloem collapses in the manner described.

This argument leads to the discussion of the significance of the renewal of the trace connexion with the stem. Is that renewal due to any influence of the leaf, or is it merely the result of the stimulus which the 'armpit' cambium receives from the growing shoots above? It is significant that this renewal of tissue gets less marked as the trace approaches the base of the leaf, and it must also be pointed out that in the conifers all the cambium is very closely connected—there is no wide leaf gap as in the dicotyledons, so that the trace cambium is not nearly so isolated as in the latter group.

Furthermore, there is the peculiar condition of Araucaria. As the renewal of trace connexions in this genus continues long after the leaf has fallen it certainly appears as if the production of xylem in that part of the trace lying in the stem was directly due to the influence of the shoot above—the absence of leaves permits of no other explanation.

Altogether the evidence bearing on the part played by the old leaf in the renewal of the trace in the conifer is rather conflicting, but it seems to the writer that the whole question depends on the condition of the trace cambium in the stem cortex. As long as the leaf remains alive that cambium also remains alive and is capable of response to the seasonal stimulus initiated by the growth of the buds in the upper part of the twig. This response takes the form of renewed division in the 'armpit' region of the cambium, producing the curved tracheides which form the connexion between the leaf trace and the stem wood. When the leaf dies, or is shed, then the cambium normally becomes inactive right back to the surface of the stem wood, so that when the next stretching and breaking occurs no new tracheides are interposed and very soon the end of the old trace is covered by an unbroken surface of stem wood. In the case of Araucaria the leaves live for so long that a stout peg of wood is left when the remainder of the trace dies, and it is quite possible that this is so well established that it cannot be buried and the continued production of the curved tracheides is merely due to the deformation of the stem cambium passing over or round this stump of trace. In the present work the writer has only been able to dissect stems actually bearing leaves, or having only just shed them, and has not examined any of the long-persistent traces mentioned in the literature; but these traces have been figured in various cases with the trace broken just outside the surface of the stem wood; the stump is not buried.

In both dicotyledon and conifer one cannot escape the obvious conclusion that the old leaves are almost entirely negligible in connexion with the renewal of growth in the tree. In all cases where any new vascular tissue has been produced in the leaf it has followed the inception of cambial activity by the growth of the buds above, and in the one instance where these have been removed there has been no renewal of cambial activity in stem or leaf. Hence it would seem that apart from the intervention of wound stimuli, which are not now under consideration, renewal of growth is not initiated in an adult, fully differentiated organ, though the latter may exhibit further vascular growth. This further activity in the adult structure normally needs the participation of a living meristematic layer, and if the cambium becomes incapable of further division then the production of vascular tissue is brought to a standstill, from which it cannot be restarted.

Moreover, the facts at present put forward raise the whole question of the usefulness of these old leaves in the physiology of the tree. Blackman and Matthaei (1903) say that two-year-old evergreen leaves have a low rate of assimilation even in July and August, and the anatomical evidence in the evergreen dicotyledons suggests that the leaves are incapable of translocation and therefore cannot even be used for storage. In the conifers it has been mentioned that active phloem is present in the leaves for two or three years, and the old leaves of these plants are found to be full of starch, but active growth of the new shoots may be taking place when none of the stored material has gone from the leaves.

Apart from this possible exception, it cannot be said that old evergreen leaves play any part in the constructive metabolism of the tree, and from the present facts it would appear that they should be regarded as inactive survivals rather than active agents in the progress of growth.

Finally, it must be said that it is evident from the cases of Arbutus, Rhododendron, &c., that particular instances will arise which do not conform to the general rule indicated. Such instances may be the result of either unusual conditions in the environment or of an abnormal response by the plant to the existing conditions.

SUMMARY

1. In the evergreen dicotyledons growth in size of the leaf is completed during the first year in the majority of cases. An exception is found in the lammas growth leaves of Rhododendron.

2. The production of second-year xylem is found frequently in *Arbutus Unedo* and occasionally in *Prunus Lauro-cerasus* and *Rhododendron Ponticum*, but is exceptional, as it occurs in none of the other species. In no instance is second-year phloem production recorded.

3. In no evergreen dicotyledon examined has the trace xylem maintained continuity with any stem wood more than one year old.

4. In the conifers, with the one partial exception of Araucaria, xylem is

formed only rarely after the first year, but phloem production continues at a normal rate for two or three years.

5. The exceptional features associated with the leaf of Araucaria are described.

6. In all the conifers examined the trace connexions with the successive annual rings of wood are very prominent.

7. Leaf persistence, and further vascular development in the leaf in both conifer and dicotyledon evergreen are discussed in relation to further radial growth in the axis.

In conclusion the writer wishes to express his thanks to Professor J. H. Priestley for his help and criticism in the preparation of this paper, and to the Earl of Stair for his kindness in allowing the writer to take specimens from the magnificent trees of Araucaria on the estate at Castle Kennedy.

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Chromosome Studies in Hyacinthus orientalis L.

I. The Somatic Chromosomes[1]

BY

S. P. NAITHANI

With Plate IV and twenty Figures in the Text

INTRODUCTION

ALTHOUGH the family Liliaceae has given so much information concerning the structure of the chromosomes and various other cytological problems, *Hyacinthus orientalis*, a member of the family, has received relatively little attention from the cytologists.

Among the earlier workers, Hyde (1909) studied the reduction division in the anthers and described a continuous spireme which shortens and twists into eight loops radiating outward towards the nuclear membrane, the crossing threads forming a central knot. These eight loops she found to break apart into eight bivalents at the first maturation division. In the variety *albullus* Müller (1912) observed eight long V-shaped, four medium, and four short J-shaped chromosomes in the root-tips. He found the chromosomes arranged in pairs throughout the somatic division, the pairing being especially noticeable in the polar view of the telophase. Carruthers (1921) saw fractures or constrictions of the chromosomes into separate segments in the somatic division of the same variety, but no pairing of the somatic chromosomes. De Mol, working on different varieties, described eight long, four medium, and four short chromosomes in the root-tips of diploid varieties and twelve long, six medium, and six short in the triploids. By examining a number of varieties he showed nineteen diploid plants, four triploid, five hypotriploids, and five hypertriploids. He (1926) suggests a relationship between the number of chromosomes and the number of nucleoli; diploids having usually two nucleoli of equal size while triploids generally have three nucleoli of equal size, the highest number of nucleoli developed in aneuploids being three or four, depending on the number of chromosomes possessed by the variety. Constrictions are not shown in his figures.

Belling (1924, 1925, 1929) describes three types of chromosomes in the pollen grains of 'Yellow Hammer', a diploid variety: (1) two pairs long with median constrictions, (2) one pair medium with subterminal constrictions, and (3) one pair short, also with terminal constrictions. He finds the pairs

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identical and assumes that pairing may have arisen in the history of the species by doubling. He concludes that this clone is possibly either tetraploid or still shows signs of descent from a tetraploid ancestor. The same explanation is put forward in the case of a triploid. He therefore calls a diploid variety a 'double-diploid' and a triploid variety a 'double-triploid'. He further describes the configurations of the chromosomes in certain diploids and triploids at the first metaphase. Darlington (1926) describes four types of chromosomes in the pollen grains of *H. orientalis*. He finds that one of the four V-shaped chromosomes has a secondary constriction, and on this account disputes Belling's contention of the doubling of the chromosomes. He further (1929) describes the method of pairing in certain trisomic and tetrasomic varieties. Stone and Mather (1932) find a direct relationship between the number of chiasmata and the length in the three types of chromosome.

Since the spiral structure of the chromosomes was first described by Baranetzky (1880) in the pollen mother-cells of *Tradescantia Virginica*, similar structures have been observed by several investigators recently in different plants and animals, especially in species having larger chromosomes. There are various points on which diversity of opinion still exists. Hyacinthus has fairly large chromosomes, so the present study was undertaken with a view to shedding some further light on the various problems confronting cytologists, with special reference to the structure of the chromosome.

MATERIAL AND METHODS

Bulbs of different varieties of *Hyacinthus orientalis* were obtained from Messrs. Sutton & Sons, Reading, and Messrs. Alexander & Brown, Perth. Some bulbs were grown in clean moist sand in pots and others in jars of water. Root-tips were usually fixed between 9 a.m. and 1 p.m. Portions about a quarter of an inch long were cut in the fixing fluid by means of a sharp razor-blade. In some cases they were split longitudinally into two halves to ensure quick fixation. An exhaust pump was invariably used to secure rapid penetration of the fixing fluids. The following were some of the fixatives used:

1. Fleming's solutions, medium and strong (La Cour, 1931)
2. Benda's fluid ,, ,,
3. La Cour's 2BE and 2 BD ,, ,,
4. Smith's S_1 and S_2 (Huskins and Smith, 1935).
5. Merkel's fluid (Chamberlain, 1928).
6. Nebel's fluid (Nebel, 1933).
7. Maeda's modification of Navashin's fluid (Maeda, 1930)
8. Belling's modification of Navashin's fluid (Belling, 1928).

Pretreatments as recommended by Sharp (1929) and Nebel (1933) were also tried, but they gave no satisfactory results.

Of the various fixatives used, the osmic mixtures gave good results, but Benda's fluid was found by far the best for the study of the chromosome structure. Merkel's fluid was inferior to osmic fluids, and Navashin's fluid did not prove satisfactory for the structure of chromosomes.

Root-tips were fixed for twenty-four hours in osmic solutions, washed in tepid water for three to four hours, changing the water every half-hour, and passed through grades of alcohol beginning from $2\frac{1}{2}$ per cent. In Navashin's fluid the root-tips were fixed for four hours, washed in 50 per cent. alcohol, and then graded up. Material was cleared in chloroform and embedded in paraffin wax according to La Cour's (1931) schedule. Microtome sections of various thicknesses ranging from 5 to 20 μ were cut to suit the requirements. Sections were stained mainly in Newton's modification of iodine-gentian violet. For comparison, sections were also stained with iron-alum-haemotoxylin according to the short methods recommended by Kaufmann (1926) and Hedayetullah (1931). But these sections were much less clear in detail than the gentian violet preparations. Feulgen's reaction was also used.

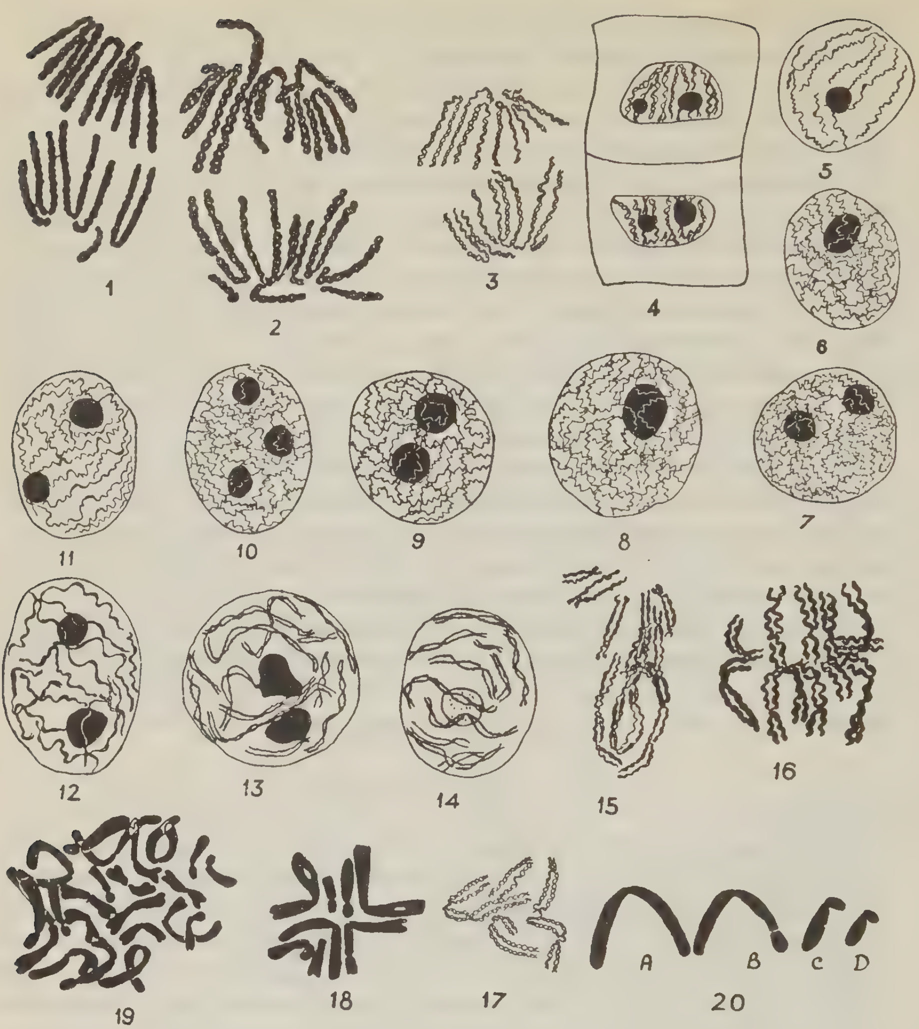
In view of the fact that Kuwada and his collaborators were able to see the spiral structure of the meiotic chromosomes clearly in aceto-carmine, smears of fresh root-tips were examined in Belling's iron-aceto-carmine. The internal structure of the chromosomes was better observed in such preparations than in sections. The following method was adopted: Fresh root-tips were cut and put on a slide. They were then crushed between two slides and stained with iron-aceto-carmine. After mounting, the slide was gently heated four or five times and the cover-slip was lightly pressed to spread the tissue farther. The preparation was ready for examination after three hours.

OBSERVATIONS

Anaphase.

At the commencement of anaphase the chromosomes arch towards the poles, showing V- and J-shaped appearances according to the positions of the constrictions (Text-fig. 1). The separation of the chromosomes at this stage is regarded by some as a result of 'pull' from the poles and by others as due to a repulsion of the chromatids. It is highly probable that the combined effect of both these forces is responsible for the separation of the chromosomes.

The structure of the chromosomes at the anaphase stage has been variously described by the different observers. Some describe them as uniformly stained bodies showing no internal structures, others have described chromatic spirals. Some observed a single spiral, while others have seen them as consisting of two spirals either running parallel or intertwined. This variability in opinion is explicable on consideration of the fact that at this stage the matrix stains deeply, and so the appearance of the chromosomes is different in differently stained preparations. In the present material in deeply stained preparations the internal structure of the chromosomes is more or less obscured and they show corrugated surfaces (Text-fig. 1). But by a longer destaining process it is possible to see that they are composed of two threads frequently twisted about one another (Text-fig. 2). Their double nature



TEXT-FIGS. 1–20. Text-figs. 1–20 were drawn with the aid of Reichert camera lucida at table level using a Busch $\frac{1}{12}$-inch oil immersion N.A. 1.30, Zeiss compensating oculars K12 (15×) or K20× and immersed aplanatic condenser N.A. 1.40.

Magnification of Figs. 3, 14, and 17 × 2100. Magnification of the rest of the figures × 2870.

Fig. 1. Anaphase chromosomes, deeply stained, showing corrugated appearances. Fig. 2. Anaphase chromosomes, partly destained to show each chromosome with two intertwined chromonemata. Fig. 3. Anaphase, iron-aceto-carmine preparation, the two intertwined spiral chromatids clearly visible. Fig. 4. Early telophase, relaxation of the spirals showing intertwined chromonemata. Fig. 5. Late telophase, expansion of the spirals, forming larger loops. Figs. 6–10. Resting stage, spirals farther drawn out. Figs. 6 and 7, diploid, with one big or two small nucleoli. Figs. 8–10, triploid, with one big to three small nucleoli. Figs. 11–13. Successive stages of prophase. Relic spirals disappearing and the new internal spiralling developing. The number of interchromatid coils is also diminishing. Fig. 14. Late prophase in iron-aceto-carmine showing the new coiling of each chromonema. Fig. 15. Prometaphase, the smaller chromosomes have separated from each other except at the spindle

becomes very clear when the root-tip smears are examined in iron-aceto-carmine (Text-fig. 3). Plate IV, Figs. 1 and 2 are photomicrographs of the anaphase stage seen in aceto-carmine. In the chromosomes indicated by arrows their double intertwined nature is clearly shown. In some cases the bifid ends are also visible (Text-fig. 19).

As the chromosomes reach the poles they clump together (Pl. IV, Fig. 2), forming a more or less deeply staining chromatic mass. Grégoire was the first to describe this stage, and he called it the *tassement polaire*. Kaufmann (1926), Sharp (1929), and others have observed it in their material. Overton (1922) finds no clumping in his material fixed with Merkel's fluid and so he regards it as a fixation artefact. Hedayetullah (1931) finds it a constant feature in Narcissus, even when fixed in Merkel. In Hyacinthus this stage is invariably present. It is quite probable that it may become exaggerated by fixation. The separate appearance of chromosomes is not wholly lost, but their structure is generally obscured. Sometimes, however, chromosomes showing spirals can be distinguished.

The anaphase chromosomes have often been regarded as composed of two substances, chromatic and achromatic. Kaufmann (1926) regards these as synonymous with oxychromatin and basichromatin. Sharp (1929) and others find the same distinction. These two substances have a more nearly equal affinity for the stains at this stage than during other stages. Darlington (1935*b*) finds no valid grounds for making a distinction between a staining 'chromonema' and a non-staining 'matrix' lying around it. He expresses the view that 'protoplasm, like other protein-water systems, has the property of separating into parts which differ in water content and other associated properties under the action of external or internal stimuli', e.g. by dehydrating fixatives. He compares the separation of the chromosome into stainable and non-stainable parts with that of milk separating into curd and whey, whose water content depends on the stimulus effecting the separation. He, therefore, considers the matrix an artefact.

The existence of the matrix has been demonstrated by various investigators using different fixatives. Kuwada and his collaborators see its presence in their aceto-carmine preparations. Sakamura (1927) finds that when the chromosomes are treated with boiling water, the matrix becomes dissolved, leaving the chromonema spirals. Bělař (1929), by comparing living with fixed and stained cells, observes its existence in the staminal hairs of Tradescantia. La Cour (1935) shows that by pretreatment of the heterotypic metaphase

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attachment region. Fig. 16. Metaphase, chromosomes arranged at the equatorial plate. Fig. 17. Metaphase in iron-aceto-carmine showing the quadripartite nature of the chromosomes; the daughter chromonemata intertwined in each sister chromosome. Fig. 18. Metaphase of the first division in the pollen grain, showing the eight chromosomes of four different types. Fig. 19. Metaphase chromosomes in the root-tips of the variety *Captain Byoton* with twenty-two chromosomes. Fig. 20. A, long, V-shaped with median constriction; B, same as A with a secondary constriction; C, medium, J-shaped with subterminal constriction; D, same as C but shorter.

chromosomes with ammonia and various acids before fixing in osmic mixtures, only the chromatic spirals are visible. This suggests the presence of matrix which has become dissolved by these agents. The above facts go to prove the definite existence of the matrix. If it is an artefact it is a 'characteristic artefact' as are chromosomes, and it bears a certain relationship with the structure of the living chromosomes. Moreover, if the chromosomes behave like a protein-water system, as Darlington believes, and separate into stainable chromonema and non-stainable matrix, then such a separation should be a constant feature throughout the mitotic cycle, unless, of course, the constitution of the chromosomes changes.

Telophase.

The telophase stage begins with the expansion of the chromosomes after the polar clumping. A wall is laid down at the equatorial plate separating the two daughter cells. Around each group of chromosomes a limiting membrane is formed separating it from the cytoplasm (Text-fig. 4). The spiral structure of the chromosomes is clearly revealed at this stage, due to the relaxation of the coils and because the matrix becomes diffuse and loses its staining capacity. The two chromatids thus become visible, showing at places clear areas which give the appearance of the so-called alveoles of Grégoire and his school. As the telophase advances the chromosomes become further unravelled and their spiral nature becomes still clearer. The threads become thinner and assume an irregular spiral form of greater diameter (Text-fig. 5) which has been called a 'super spiral' by Darlington (1935*a*). At late telophase due to further uncoiling of the spiral threads, the two chromatids come so close together that their double nature is completely obscured.

The anastomosing strands described by various investigators as connecting the different chromosomes and forming ultimately a reticulum in the resting nucleus are no doubt artefacts. In preparations which were adequately fixed, and also in those examined in iron-aceto-carmine they were absent.

In the meantime a nucleolus or nucleoli have become reconstituted. At first they are smaller, but later they assume their normal size (Text-fig. 5). No evidence could be found of the way they arise, but it is highly probable that their appearance has some connexion with the disappearance of the matrix as has been suggested by various observers. Certain refractive bodies were visible embedded in the nucleoli.

Merriman (1904) and Nebel (1933) observe the chromosomes quadripartite at this stage. No such extra-split was seen in Hyacinthus.

Interphase and resting stage.

Actively dividing cells rapidly pass on to the prophase stage without the intervention of a definite period of rest. Lundegårdh (1912) was the first to use the term 'interphase' for this short stage of the nucleus between two successive mitotic divisions in rapidly dividing tissue. Less actively dividing cells, especially those in the older portion of the root, pass to the resting stage. In early stages the chromonema spirals are much loosened and are partly drawn out in zigzag threads. The knotted appearances observed at this stage are the optical images of the spirals, as has been pointed out by Bělař (1929). With the advancement of the stage the spirals are farther drawn out and the elongated threads ramify the entire nuclear area, rendering it impossible to trace them throughout their length, but a few free ends here and there indicate their individuality. The threads at this stage are at their maximum length and minimum thickness (Text-fig. 6). There is no evidence of any matrix at this stage.

Accurate observation of this stage is very difficult, and most of the opinions so far advanced are largely based on deductions from a comparison with the preceding telophase and the prophase which follows. The threads are very delicate. Their viscosity is at its minimum and so, due to improper fixation, they become distorted, giving the granular and reticulate appearances observed by various authors. Bělař (1929) and Kuwada and Nakamura (1933) do not observe any reticulate structure in the living nucleus of the staminal hairs of Tradescantia. Nor was it seen by several other observers in properly fixed preparations.

There is either one big nucleolus or two small ones in diploid varieties at this stage (Text-figs. 6, 7). In triploid varieties the number of nucleoli varies from one big to three smaller ones (Text-figs. 8, 9, 10), but there are never more than two in a diploid or three in a triploid variety. This is in accord with De Mol's (1926, 1929) observations.

Prophase.

As the prophase begins, the 'old spirals' of the preceding telophase stage reappear (Text-fig. 11). Darlington (1935) calls them the 'relic spirals'. The threads become distorted into irregular spirals of larger loops (Text-fig. 12). Sharp (1929) does not show these larger loops in his prophase figures (Sharp, 1929, Figs. 19–28), but shows only the straight arrangement of the threads. At early prophase the threads are still so closely approximated with each other that their double nature is indistinct. But as the prophase advances there is a gradual contraction in length and thickening in diameter of the threads. Their dual nature has now become clearer (Text-fig. 13). The explanation offered for this contraction and thickening is that the chromosomes are undergoing new internal spiralization. This spiralling begins with the commencement of the prophase stage. In sections of fixed material it is not clearly made out, and the closely contracted spiral thread presents a chromomeric appearance. This is really due to the optical effect produced by observing such a fine spirally twisted thread at one focus. If, however, the focus is changed and the thread followed up carefully, its continuity in spirals is clearly noticed and the chromomeres are found to be merely optical illusions.

This new spiralling is rendered distinctly visible in preparations in iron-aceto-carmine (Text-fig. 14).

The fact that the finely coiled spirals are visible in iron-aceto-carmine preparations and that they appear as granules in sections of fixed material leaves no doubt that the so-called chromomeres described by various investigators in fixed preparations represent the twists of the spirals.

The internal spiralling leads to two results. Firstly, it shortens the chromosomes. This shortening does not necessarily mean any decrease in the actual length of the chromonema threads. The gene string maintains approximately the same length at all stages in the chromosome cycle, as Bridges (Alexander, 1928) assumes. It is the chromosome which lengthens and shortens. The second effect is that the two chromonemata of each chromosome are gradually unravelled, with the result that the chromosome appears to be composed of two intertwining spiral threads. During prophase, as the new or minor spiralling develops, the relic spirals gradually disappear. As the stage advances the intertwinings between the two chromatids, which may be called the interchromatid coiling, decrease in number as the relic coils lessen (Text-figs. 13, 14). By prometaphase and metaphase the relic spirals have completely disappeared and the interchromatid coiling has decreased considerably. In smaller chromosomes the two chromatids are completely separated, remaining attached to one another only at the spindle attachment region. The longer chromosomes remain still intertwined with one another (Text-fig. 13). At the prometaphase the split for the next division becomes visible (Text-fig. 15). The split takes place along the longitudinal axis of the spiral chromonema. The matrix now increases its affinity for the stain, rendering the internal structure difficult to observe.

The nucleoli are spherical in shape in earlier stages, later they become irregular and assume amoeboid appearances (Text-fig. 13).

Metaphase.

At the end of the prophase the nuclear membrane disappears and the chromosomes arrange themselves at the equatorial plate (Text-fig. 16). Each half-chromosome now consists of two spirals with regular parallel coils. In longer chromosomes the interchromatid coiling persists, while in smaller ones it has disappeared. The matrix is deeply stained and then it becomes difficult to observe the internal structure of the chromosomes, but corrugated appearances indicate their spirality (Text-fig. 16). In iron-aceto-carmine the coiled nature of the chromosomes is very distinct (Text-fig. 17). Here each half-chromosome is seen to be composed of two chromatid spirals twisted about one another (Text-fig. 17 and Pl. IV, Fig. 5). The arrows in the photomicrograph show the four ends of the chromosome (Pl. IV, Fig. 5).

The nucleoli by this time have disappeared. It seems highly probable that the increased chromaticity of the chromosomes is due to the passing of the nucleolar material to them, as pointed out by various investigators.

Sixteen chromosomes are arranged at the equatorial plate in a diploid variety. They fall into four types, as represented in the diagram (Text-fig. 20).

They have been described by Darlington (1926). Their shapes vary according to the position of the attachment constriction. A is a long chromosome, V-shaped with a median constriction. B, also V-shaped, can be distinguished by the presence of a secondary constriction in addition to the median. C is a medium-sized, J-shaped chromosome with a subterminal constriction. D is the short chromosome, also J-shaped with a subterminal constriction. These chromosome types are more clearly discerned during the first division of the pollen grain (Text-fig. 18). De Mol (1921) and Belling (1924, 1925) describe only three types of chromosomes. De Mol does not show the constrictions in his figures, and Belling could not determine whether the secondary constriction was constantly present in type B.

The chromosome number of the variety *Captain Byoton*, not previously examined, has been determined. It is an aneuploid possessing twenty-two chromosomes (Text-fig. 19). The complement is composed of fifteen long, three medium, and four short chromosomes.

In none of the stages were the chromosomes seen arranged in pairs as Müller (1912) recorded in *H. orientalis*. Their arrangement is at random. It may be that in some cases in this random arrangement some pairs may lie close together.

DISCUSSION

Structure of the chromosomes.

The study of the chromosomes of *Hyacinthus orientalis* seems to clear up several contradictions in the interpretation of chromosome structure by various investigators. In the first place it is seen that the chromosomes exist as spiral threads throughout the somatic division. The earlier alveolar conception foreshadowed by van Beneden and formulated into a theory by Grégoire (1906, 1907, 1912) and his school has to be discarded. It has been shown that the so-called alveoles represent the translucent spaces between the two intertwined chromatids.

At present there are two main theories of chromosome structure in vogue. According to the chromomere theory the chromosomes in prophase are made up of granules or chromomeres strung together on a fine connecting thread. According to the other, the chromonema theory, the core of the chromosome contains a continuous thread or chromonema, which takes a spiral form in various stages of mitosis. Both theories have strong supporters. In the course of the discussion it will be shown how the chromonema spirals give the appearance of chromomeres.

The chromomere theory is the oldest and was introduced by Pfitzner in 1880. He suggested that the chromosome is made up of a row of granules embedded in an achromatic matrix. These granules were called 'Pfitzner's granules'. Fol in 1891 used the term chromomere as equivalent to chromosome.

But now the granules alone are known as chromomeres. Wilson (1896) observed still smaller bodies composing a chromomere. Eisen (1900) gave the name 'chromioles' to those smallest visible particles. Strasburger (1907, 1911) observed definite morphological units or chromomeres arranged in a linear order along the thread. In Primula, Digby (1912) described the chromomeres as formed by stringing together of homogeneous beads of chromatin. Wenrich (1916) demonstrates the presence of chromomeres of different sizes at fixed positions on the chromosome in Phrynotettix. Sands (1923) observed chromomeres of different sizes and shapes by treating the pollen mother-cells of *Tradescantia Virginica* with aceto-carmine. Belling (1928, 1931) remarked that paraffin sections are not good for demonstrating the chromomeres. He found smear preparations best to show chromomeres. Working on liliaceous plants, he has not only seen chromomeres but has been able to estimate their number.

Babcock and Clausen (1929), working on the sporocytes of Crepis, figure chromomeres in the middle prophase, but show only a spiral chromonema whose coils may appear superficially like large chromomeres at the late prophase. Hedayetullah (1931) in Narcissus and Perry (1932) in Galanthus observe chromosomes chromomeric at some stages and chromonemic at others. Darlington (1935*a*) in Fritillaria and Huskins and Smith (1935) in Trillium describe chromomeres in the prophase stages.

Such chromeric appearances have been observed by various other workers. Kaufmann (1926) interprets them as representing 'only doubleness or greater amount of thread surface'. Smith (1932) thinks 'that the granules on the network of the resting nucleus apparently correspond to points at which the chromonemata of each chromosome are in contact with each other'. O'Mara (1933) and Iwata (1935) explain the granular appearance as due to the fact that only the crests of the wavy chromosomes are in sharp focus. Koshy (1933) regards the appearance of granules at the resting stage as due to the twists and bends of the zigzag threads.

It is to be noted that these granules have been described at the resting, early, and middle prophase stages. At late prophase and after, the chromosomes are usually described as consisting of spirals. As pointed out above, their appearance at the resting stage is an artefact. The chromonema threads at this stage are extremely delicate. Their viscocity is at its minimum and so they are too diffuse to resist the effect of the various fixatives. Hence the thin threads become broken up into granules when not suitably fixed. Their appearance at the early and middle prophase is due to the optical effect of the new coiling of the chromonema thread. In these stages this coiling is very fine, and so in section of the fixed material these contracted coils appear as chromomeres. But in aceto-carmine preparations their spirality can be clearly seen. In late prophase and after, these fine coils assume a larger diameter and so they appear as regular spirals.

The chromomeric appearances could neither be due to the doubleness of the thread nor the greater amount of thread surface, as Kaufmann suggests, since they are visible in each individual thread in the middle prophase when the two threads are quite separate, and are intertwined at longer intervals and not closely intertwined as Koshy (1933) finds in Allium. Similarly, Smith's suggestion that these granules which he sees in the resting stage correspond to points at which the chromonemata of each chromosome are in contact with each other can be disregarded. It is then clear that the so-called chromomeres are the optical images of the finely coiled spirals greatly exaggerated by the fixatives. Careful examination of the granules in the fixed material shows that the granules themselves are spirally arranged, thereby suggesting the spirality of the thread.

The chromonema theory is more modern and is supported by many recent cytologists. Baranetzky (1880) examined the sporocytes of *Tradescantia Virginica* and noted the spiral structure of meiotic chromosomes. Janssens (1901) observed finely coiled threads in the telophase chromosomes of the spermatogonia of Urodeles. This conception was embodied in the chromonema hypothesis developed especially by Bonnevie (1908, 1911) and Vejdovsky (1911, 1912). Bonnevie concluded from her studies on Ascaris, Allium, and Amphiuma that each chromosome in the telophase develops an endogenous spiral thread which persists through the interphase and emerges from the chromosome in the following prophase. De Horne (1911) found the telophase chromosomes of Salamandra and Allium to be formed of two interlacing spirals. Vejdovsky (1911–12) was the first to apply the term 'chromonema' to the spiral filaments. He finds the spiral to arise much earlier than does Bonnevie. It first develops in the prophase within the yet unsplit chromonema, and as the latter splits the new chromonema becomes broken up into irregular rings or strands, which in some undetermined manner form a continuous spiral again in the telophase.

Such chromonemata have been observed by Kaufmann (1925, 1926, 1931) in Tradescantia, Podophyllum, Allium, and Iris; Sharp (1929) in Trillium, Vicia, Podophyllum, Allium, and Tradescantia; Telezyneski (1930, 1931) in Tradescantia and Haemanthus; Hedayetullah (1931) in Narcissus; Nebel (1932) in Tradescantia; Perry (1932) in Galanthus; Janaki-Ammal (1932) in Nicandra; Smith (1932) in Galtonia; Koshy (1933) in Allium, and Hoare (1934) in Scilla. These workers make no mention of the finely coiled new spirals in the prophase. The spirals observed by them presumably represent the relic spirals of the author. Upcot (1935) was able to see the new coiling directly in the prophase stage of the pollen-tube division of Tulips examined in aceto-carmine. They were observed by Vejdovsky (1911–12) at metaphase in Ascaris. Darlington (1935*a*) obtained indirect evidence of their spiral nature at telophase in Fritillaria.

Heitz (1933, 1934), Painter (1934), Metz and Gay (1934), Metz (1935 *a*, *b*) and other recent animal cytologists describe the chromosomes of the salivary glands of Drosophila and Sciara as consisting of very conspicuous bands or discs of deeply staining material alternating with clear areas. But Kaufmann (1931) in Drosophila, and more recently Sinoto and Yuasa (1935) in Drosophila and Sciara have observed spiral structure in salivary chromosomes. Kaufmann remarks that the discoid structure is the result of an inadequate technique and concludes that the spiral bands present a more accurate picture of the normal arrangement of the chromatic material, while Sinoto and Yuasa describe the chromosomes of salivary glands to be doubly coiled. They even suggest the existence of a tertiary coiling.

In view of this evidence and that produced in the present study, it appears to be proved beyond doubt that the chromosomes exist as spirally coiled threads throughout the mitotic division, and the so-called chromomeres are in this case optical images of the finely coiled spirals greatly exaggerated by inadequate technique.

Duality of chromosomes.

In tracing the history of the chromosomes in the mitotic division of Hyacinthus it has been demonstrated that they are double at anaphase, telophase, and prophase and quadripartite at prometaphase and metaphase. Fraser and Snell (1911), Schustow (1913), and Sarbadhikari (1924) have recorded the duality of the telophase chromosomes. The work of Merriman (1904), Taylor (1925), Kaufmann (1926), Sharp (1929), Telezynski (1931), Hedayetullah (1931), Perry (1932), Smith (1932), Koshy (1933), and Hoare (1934) has now firmly established the fact that the anaphase chromosome is double. Hunter (1934) observes the quadripartite nature clearly in end views of the metaphase chromosomes in root-tips of Anthoxanthum. In the pollen grains of Trillium, Huskins and Hunter (1935) find the metaphase chromosomes quadruple. Sax's (1935) photomicrograph of the metaphase chromosomes in the pollen grain of *Rhoeo discolor* leaves no doubt about their quadripartite nature. Darlington (1926) described a quadruple structure of the metaphase chromosomes in the pollen grains of Hyacinthus and Scilla, but now he considers such observations as optical illusions due to the 'hollow' nature of the chromosomes. My studies on the somatic chromosomes of Hyacinthus furnish cogent evidence in support of the fact that the chromosomes are double at anaphase and quadruple at metaphase.

Division of the chromosomes.

Having established the double nature of the chromosomes at all stages except prometaphase and metaphase, let us examine where splitting takes place. Cytologists in general are now agreed that the split in the chromosomes takes place longitudinally, which is a plausible conclusion in view of their genetic continuity. But there are still considerable differences of opinion with regard to the stage at which this split occurs.

Flemming (1880) suggested that the split of the chromosome took place at the metaphase of the same division at which they separate. Grégoire and his school found the splitting occurring at prophase, brought about by the fusion of the longitudinally alined central series of alveoles formed in the previous telophase. Müller (1912) expressed the opinion that the portion of the thread between the chromomeres split first, followed by the division of the chromomeres, thus completing the division. Gates (1912) and Sharp (1913) described the splitting as a prophasic phenomenon.

Lundegårdh (1911), Fraser and Snell (1911), De Horne (1911), Digby (1910, 1912), Schustow (1913), and Sarbadhakiri (1924) maintained that the split in the chromosomes takes place in the preceding telophase.

These older ideas have now been given up, and in recent years two main view-points have been put forward regarding the stage at which the mitotic chromosomes divide. According to one, they divide at the metaphase or prometaphase of the previous mitosis. This view is largely the outgrowth of the work of Kaufmann (1926), Sharp (1929), Perry (1932), Smith (1932), Koshy (1933), Hoare (1934), Hunter (1934), Huskins and Smith (1935), and Sax (1935), who claim to have seen the chromosomes at anaphase as double and point out that the split takes place at prometaphase. Darlington and his co-workers deny the accuracy of these observations and affirm that the chromosomes divide (invisibly) during the resting stage. Mather and Stone (1934), by irradiating the root-tips of Crocus, obtained 'chromosome breaks' and state that their experiments support Darlington's contention. Huskins and Hunter (1935) have contradicted their results and found that 'chromatid breaks' have occurred in the chromosomes of *Trillium erectum* by the effects of X-radiation and argue that the chromosomes split in the prophase of the division before that at which the chromatids so formed will separate. White (1935), studying the effects of X-rays on mitosis in the spermatogonial division of *Locusta migratoria* L., encountered both 'chromosome' and 'chromatid' breaks. He thereby suggests that breakage can take place in both chromatids at the same level, a possibility which was rejected by Mather and Stone. My work on Hyacinthus chromosomes supports the view that the chromosomes divide at the prometaphase of the somatic division and the sister chromatids separate at the anaphase of the succeeding division.

Moreover, the idea that the division of the chromosomes takes place at the resting stage is inconceivable in view of the fact that in actively dividing meristems there is no definite period of rest. There is another fact which needs consideration. The split of the chromonema thread would necessitate the duplication of the materials composing it. During the prophase stages there is a gradual increase in the volume of the cell and nucleus. This clearly suggests that there is a definite increment in the cytoplasm and nucleoplasm. In view of this fact it is highly probable that there is a gradual increment of the chromatin during prophase. The duplication of the chromonema may not take place at the same time throughout its whole length. It is likely that it takes place at somewhat different times in different places. Also, the duplication of the units composing the chromonema micellae may be interdependent, i.e. the formation of one unit may depend on the previous formation of another either of a different or similar type. The duplication presumably is complete before metaphase, at which stage the split becomes clear and the nucleus attains its maximum volume. From this physiological aspect of the question, therefore, the idea of the prometaphasic split appears to be quite tenable. The separation of these units into two chromatids probably results, as suggested by Wrinch (1934), from the surface charge becoming so great that instability results.

The line of cleavage is along the longitudinal axis of the spiral chromonema thread. Koshy (1933) finds in Allium that the daughter chromosomes at metaphase have approximately three or four times the number of twists of the parent chromosomes, from which he infers that the line of cleavage is not straight along the axis of the parent chromonema but takes a spiral course in its formation. No increment in the number of twists in the daughter chromosomes was observed in Hyacinthus.

Mechanism of the unravelling of the chromatids.

In the history of the mitotic cycle of the chromosomes in Hyacinthus we find that at early prophase the coiled chromatids are closely intertwined around each other. The closeness of the intertwining decreases as the prophase advances; that is, the number of turns round each other become progressively less until at metaphase the two half-chromosomes become practically separated from each other and lie as two parallel coiled threads, remaining attached to each other only at the spindle attachment region.

The following observations seem to offer an explanation for the separation of the two chromatids. With the commencement of the prophase the chromonema threads begin to undergo new spiralling, which gradually shortens and thickens the chromosomes. Each chromonema thread coils in a direction opposite to that of its partner and away from the axis of the chromosome, and they therefore tend to move away from each other. The unravelling of the two chromatids may thus be explained as due to this mode of opposite coiling of the chromonema threads. If, as suggested, this new coiling is in opposite directions in each chromonema, then the two chromatids will go on unravelling until they come to lie side by side, the one being the mirror image of the other, giving a symmetrical pattern. Kuwada and Nakamura (1935) explain the decrease in the number of twists in the chromosomes of Fritillaria as due to the new coiling of the chromonemata at early prophase. Koshy (1933) finds in Allium that the chromonema threads are closely intertwined with each other at metaphase and explains the separation of the chromosomes at this time by unwinding from the ends towards the constriction where they are held together. In Hyacinthus, however, the uncoiling of the two chromatids begins in prophase, when the new spiralling develops, and is completed in the smaller chromosomes before metaphase is reached. In the long chromosomes the uncoiling is completed during metaphase.

SUMMARY

The investigation of the somatic chromosomes of Hyacinthus shows that the chromosomes at anaphase are composed of two intertwining spirals embedded in a less chromatic matrix.

Their double nature becomes very clear at early telophase when the spirals are relaxed.

At the late telophase and resting stage the duality is completely obscured, due to the sister chromatids becoming closely approximated with each other.

The granular and reticulate appearances are regarded as artefacts.

With the commencement of prophase a new coiling begins in each chromatid, which shortens and unravels the chromatids and throws the relic spirals into coils of larger loops. This spirality is finer at early stages, later it assumes a larger diameter. It is clearly seen in iron-aceto-carmine preparations.

At prometaphase the split for the succeeding division takes place in the chromosomes. The metaphase chromosomes are thus quadripartite.

The chromomeres are shown to be an optical appearance produced by the finely coiled spirals and often assisted by imperfect fixation.

Reversal in the direction of coiling takes place only at the spindle fibre attachment.

An explanation is offered for the splitting of the chromosomes at prometaphase and the separation of the two intertwined chromatids later in the succeeding prophase.

In conclusion, I wish to express my grateful thanks to Professor R. Ruggles Gates, for his helpful suggestions and criticisms throughout the course of these investigations. I have also great pleasure in acknowledging my indebtedness to the University of Allahabad and the Chandra Ballabh Memorial Trust, Garhwal, for affording me facilities to undertake this work in the Botany Department of King's College, University of London.

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EXPLANATION OF PLATE IV

Illustrating Mr. Naithani's paper on 'Chromosome Studies in *Hyacinthus orientalis* L.'.

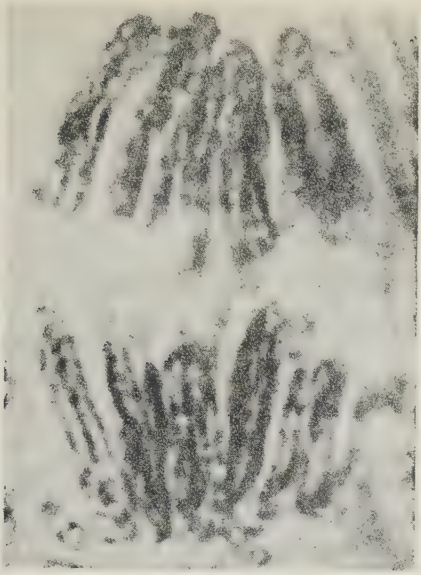
All the photomicrographs were taken from iron-aceto-carmine preparations, using Busch $\frac{1}{12}$-inch oil immersion N.A. 1.30 and Zeiss compensating ocular 6.

Figs. 1 and 2. Anaphase chromosomes showing their double intertwined nature.

Fig. 3. The 'tassement polaire' stage. The spiral nature of the chromosomes is distinct in some chromosomes.

Fig. 4. Prophase, showing the superspirals, and that each chromosome has two intertwined chromatids.

Fig. 5. Metaphase chromosomes showing their quadripartite nature.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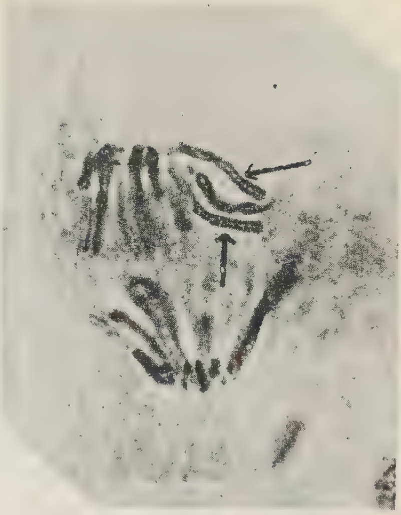
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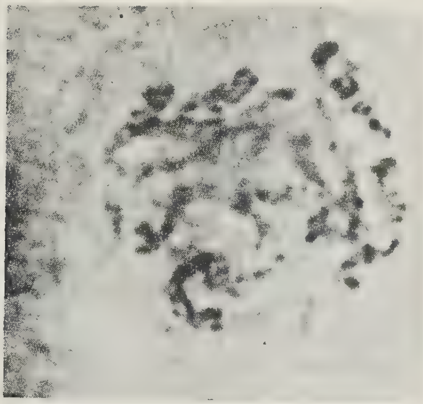
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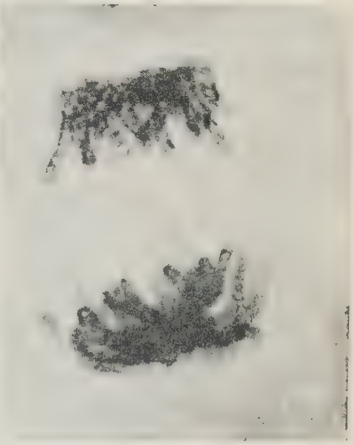
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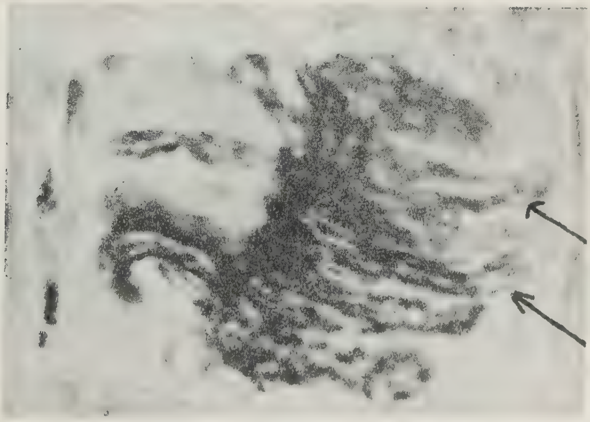
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Huth coll.

NAITHANI — HYACINTHUS ORIENTALIS.

A Study of the Effect of Blue-violet Rays on the Formation of Carbohydrates in Leaves

BY

R. H. DASTUR

AND

S. SOLOMON

(*Botany Department, Royal Institute of Science, Bombay*)

DASTUR and Mehta (Ann. Bot., xlix, 1935) have recently determined the photosynthetic activities of the leaves in lights from different artificial sources, such as an incandescent oil lamp, a 1,500-watt electric lamp, a 'Daylight' lamp, and a carbon arc lamp. These lights differ in the intensity of the blue-violet rays which are in the order: incandescent lamp $<$ electric lamp $<$ daylight lamp $<$ carbon arc lamp. Their results show that the rate of photosynthesis increases in the same order as the intensity of the blue-violet rays increases.

They also studied the rate of photosynthesis in monochromatic red light (6,200–7,000 Å.), blue light (4,000–4,720 Å.), and in white sunlight, all of equal total intensities. The results show that the photosynthetic activity is highest in white light, intermediate in red light, and feeble in the blue-violet light. They also indicated that the red region and blue-violet region of white light were both necessary for the normal photosynthetic activity.

These authors in their investigations with the carbon arc lamp, which is the richest in the blue-violet radiation of the sources of light used by them, obtained only qualitative results. They were unable to obtain quantitative results, as only one leaf at a time could be exposed to the lamp and the methods of carbohydrate estimation are not delicate enough for the determination of the carbohydrate contents of a single leaf. Experiments were therefore undertaken to obtain quantitative data of the formation of carbohydrates in leaves exposed to the arc lamp. It was also determined to test the findings of the above-named authors by adding a beam of blue-violet light to the ordinary white light from an electric lamp, and to determine the carbohydrate contents of leaves exposed to such a mixed light.

The arc lamp.

A portable reflector arc lamp made by the Strong Electric Co., Toledo, Ohio, U.S.A., was obtained working at 55 volts D.C. and at 10 amperes. The valuable features of this lamp were the following: (1) It has a small motor maintaining a constant distance between the carbon rods, thus giving a steady

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beam of light. (2) A concave mirror acts as a parabolic reflector and gives a parallel beam of light. (3) The rods burn for at least one and a half hours, so that only three changes have to be made in the course of an experiment of six hours. (The rods used were the Ship Carbons No. 24 made by C. H. Champion & Co., Ltd., London.)

As the beam of light was horizontal a mirror 12 in. × 16 in., tilted at an angle of 45° was placed in its path, so that the beam of light was reflected vertically on to the floor, providing a large enough area for potted plants to be used. A vertical glass cooler 8 in. × 8 in. × 3 in. was interposed between the lamp and the mirror to absorb most of the heat rays, and the rest were absorbed after reflection from the mirror by a horizontal cooler 22 in. × 22 in. × 3 in. filled with running water. A convex lens was placed between the mirror and the vertical cooler.

Before exposing the plants, the intensity of the light was made equal to that of the electric light, a microthermopile in conjunction with a D'Arsonval galvanometer being employed.

The mixed light.

Attempts were made to find a device by which an addition of blue-violet rays could be introduced into the beam of light from an electric lamp, so as to obtain a beam richer in the blue-violet rays. After several unsuccessful attempts to achieve this, the following arrangement of the apparatus produced a light which approached ordinary daylight in its spectral distribution. A flood-light lamp of the Novalux Projector type (General Electric Co., U.S.A.) with a 1,000-watt bulb was used. The lamp was placed on a high stool and tilted so that the beam of light fell on two mirrors made of good Belgian glass, arranged in such a manner that the light from each, after reflection from its surface, occupied the same area and superimposed on each other, at a certain distance from the floor where plants were to be exposed.

On top of an open box placed on supports beneath the mirrors were put two glass trays so that each occupied half the area open to the passage of reflected light from the mirrors. Above these two containers, and resting on supports placed on them, was put a horizontal water-cooler holding constantly circulating water. The glass of the water-cooler cut off all the ultra-violet radiations. The distance from the floor at which the plants were exposed was 1½ feet. The area of the mixed beam of light available for exposing the plants was 15 in. × 15 in. The light reflected from one mirror and passing through a glass tray below it was allowed to fall unfiltered, whereas the light reflected from the other mirror passed through a glass tray containing a copper sulphate filter which only allowed the light between 4,200–4,720 Å. to pass through. When this was superimposed on the yellowish-white light coming from the other mirror, the light assumed a sort of greyish-white colour very much like that of diffused daylight. In this way a light resembling daylight was obtained.

A very important consideration was the control of the percentage of light added from the blue-violet filter and the measurement of the intensity of mixed light in terms of galvanometric deflection. This was beset with difficulties, as in the first place, the percentage transmission of the filter was only 23·9 per cent.; and, secondly, the thermopile used in measuring the intensity of light is not as accurate for blue-violet radiations as it is for the red and yellow. In order therefore to measure the intensity of mixed light, the following procedure was adopted:—One mirror was covered with a black cloth so that when the light was switched on no light was reflected from it. The flood-light was turned on to the free mirror and the intensity of reflected light, after passing through the water-cooler and glass tray and falling on the place where the plants were to be exposed, was measured by the thermopile. This entailed the adjustment of the mirror so that the field of reflected light had a uniform high intensity of say 16 cm. The copper sulphate filter was then placed in the glass tray beneath this mirror, and taking the transmission of light of this filter to be approximately 25 per cent., the intensity of light passing through it would be equal to 4 cm. on the galvanometer scale, whereas with the thermopile it read only 2·2 cm. This mirror was covered with the black cloth and the second exposed and so adjusted that light of exactly 4 cm. deflection fell uniformly on the field covered by the light from the first mirror. This necessitated the placing of two glass plates, 2 mm. thick, on the glass tray beneath the mirror to reduce the intensity. By these means, when the black cloth was removed and both the mirrors opened to the flood-light, the intensity of the light where the plants were exposed was equal to 8 cm., though if measured in galvanometric deflections it was only 5·5 to 6 cm. The proportion of the blue-violet light to the light from the electric bulb was therefore 1 : 1, as equal intensities of both kinds were taken and combined into one. This proportion was used throughout the investigation.

As the intensity of light taken from the carbon arc lamp was equal to 6 cm. deflection on the galvanometer scale, it was essential that light of the same intensity be used in this case also, so as to get comparable results. Therefore, instead of taking an intensity of 16 cm. as above described, from the mirror, only 12 cm. were taken, thus giving an intensity of 3 cm. when the copper sulphate solution was placed in the glass tray beneath it. This meant that one more glass plate of 2 mm. thickness had to be put on the glass tray beneath the second mirror, making three glass plates in all, so as to get a light of 3 cm. intensity from it. Both the mirrors when open to the flood-light thus gave a light of 6 cm. intensity.

Potted plants were used throughout the investigation. The plants were raised from seed sown at the same time, and for experimentation plants showing similar growth were selected. They were placed in a well-ventilated dark room for forty-eight hours. At the end of this period leaves tested showed a complete absence of starch. Three plants were then exposed under the electric bulb and three under the carbon arc lamp. The leaves of another three plants were cut and killed immediately for carbohydrate determination at the beginning of the experiment.

The same methods as in previous contributions were used for the extraction and estimation of the carbohydrates of the leaves.

The results of the carbohydrate analyses of the leaves of *Helianthus annuus* L. and *Tropaeolum majus* L., exposed to ordinary electric light, mixed light, and the carbon arc lamp are given in Table I below. Similar experiments were made with *Raphanus sativus* but the data are omitted.

TABLE I

Carbohydrate Content of Leaves Exposed to Three Different Illuminations.

Helianthus annuus

	Reducing sugars.	Cane sugar.	Starch.	Total.
	Jan. 28, 1935.			
Dark	Nil	0·0143	0·0020	0·0163
Electric lamp	Nil	0·0187	0·0038	0·0225
'Mixed' „	Nil	0·0249	0·0066	0·0315
Arc „	Nil	0·0144	0·0046	0·0190
	Jan. 31, 1935.			
Dark	Nil	0·0119	0·0042	0·0161
Electric lamp	0·0039	0·0328	0·0045	0·0412
'Mixed' „	Nil	0·0751	0·0089	0·0840
Arc „	0·0035	0·0804	0·0052	0·0891
	Feb. 4, 1935.			
Dark	Neg.	0·0144	0·0067	0·0211
Electric lamp	Neg.	0·0273	0·0089	0·0363
'Mixed' „	Nil	0·0459	0·0072	0·0531
Arc „	0·0036	0·0589	0·0080	0·0705
	Feb. 8, 1935.			
Dark	Neg.	0·0154	0·0037	0·0191
Electric lamp	Nil	0·0130	0·0074	0·0204
'Mixed' „	Nil	0·0173	0·0114	0·0287
Arc „	Nil	0·0247	0·0060	0·0307
	Feb. 12, 1935.			
Dark	Neg.	0·0275	0·0068	0·0343
Electric lamp	Neg.	0·0194	0·0060	0·0254
'Mixed' „	Neg.	0·0858	0·0743	0·1601
Arc „	Neg.	0·0344	0·0099	0·0443
	Feb. 18, 1935.			
Dark	0·0030	0·0273	0·0056	0·0359
Electric lamp	0·0033	0·0249	0·0054	0·0336
'Mixed' „	Neg.	0·0416	0·0107	0·0523
Arc „	Nil	0·0292	0·0430	0·0722
	March 6, 1935.			
Dark	0·0136	0·0234	0·0056	0·0426
Electric lamp	0·0069	0·0171	0·0069	0·0309
'Mixed' „	0·0143	0·1144	0·0097	0·1384
Arc „	Nil	0·0506	0·0080	0·0586

TABLE II

Carbohydrate Content of Leaves Exposed to Three Types of Illumination.

Tropaeolum majus

	Reducing sugars.	Cane sugar.	Starch.	Total.
April 5, 1935.				
Dark . . .	Neg.	0·0188	0·0056	0·0244
Electric lamp . .	0·0036	0·0326	0·0091	0·0453
'Mixed' „ . .	0·0719	0·0735	0·0153	0·1607
Arc „ . .	0·1150	0·1757	0·0142	0·3049
April 6, 1935				
Dark . . .	Nil	0·0238	0·0129	0·0367
Electric lamp . .	Neg.	0·0281	0·0151	0·0432
'Mixed' „ . .	Neg.	0·0469	0·0164	0·0633
Arc „ . .	0·0065	0·1834	0·0254	0·2153
April 10, 1935.				
Dark . . .	Nil	0·0133	0·0072	0·0205
Electric lamp . .	0·0037	0·0292	0·0100	0·0429
'Mixed' „ . .	0·0148	0·0790	0·0141	0·1079
Arc „ . .	0·0856	0·1148	0·0180	0·2184
April 11, 1935.				
Dark . . .	0·0055	0·0244	0·0087	0·0386
Electric lamp . .	0·0362	0·0331	0·0101	0·0794
'Mixed' „ . .	0·0539	0·1002	0·0136	0·1677
Arc „ . .	0·1080	0·1086	0·0134	0·2300
April 15, 1935				
Dark . . .	Nil	0·0171	0·0084	0·0255
Electric lamp . .	Nil	0·0228	0·0100	0·0328
'Mixed' „ . .	0·0495	0·0533	0·0112	0·1140
Arc „ . .	0·1331	0·1171	0·0134	0·2636
April 19, 1935.				
Dark . . .	Nil	0·0219	0·0123	0·0342
Electric lamp . .	Nil	0·0592	0·0081	0·0673
'Mixed' „ . .	Nil	0·1106	0·0151	0·1258
Arc „ . .	0·1331	0·1171	0·0134	0·2636

Fisher's method of '*t*' was applied to the above data, and the differences between the carbohydrates formed in ordinary electric light and in the arc light are found to be highly significant; the data for *Raphanus sativus* L. are also highly significant. Similarly, the differences between the electric light and 'mixed' light are also of high statistical significance in all three plants. The differences between 'mixed' and arc light are highly significant in *Tropaeolum majus* and also in *Raphanus sativus*, but not in *Helianthus annuus*.

CONCLUSIONS

The results obtained in the above investigation are clear. More carbohydrates are formed in the leaves exposed to the carbon arc lamp than are formed in the leaves exposed to the electric lamp during the same period, all other external conditions being as uniform as possible. The greater formation of carbohydrates in leaves exposed to the carbon arc lamp means greater photosynthetic activity than in leaves exposed to the light from the gas-filled electric lamp. As the total intensities of light falling on the leaves exposed to the lights from the two sources were equal, the differences between the carbohydrate contents of the leaves exposed to the light from the carbon arc lamp and the gas-filled electric lamp can be safely attributed to the spectral differences in the two lights. As the main difference in the lights from the two sources is in the greater intensity of the blue-violet rays in the carbon arc lamp, it is safe to attribute the differences in the photosynthetic activity to the richness of the blue-violet rays in the carbon arc lamp. Thus the findings of Dastur and Mehta (1935) are confirmed by quantitative data.

When the plants are exposed to 'mixed' light by superimposing a beam of blue-violet light on that from an electric lamp, the carbohydrates formed in the leaves in such mixed light are higher than those formed in the leaves exposed to light of the same total intensity from the gas-filled electric lamp only. Though the differences in the carbohydrate contents of the leaves exposed to mixed light and the gas-filled electric lamp only are not as large as in the case of the arc and electric lights, they are, however, constant and statistically significant. By adding a beam of blue-violet light the photosynthetic activities of the leaves are increased, and thus the importance of the blue-violet rays for the photosynthetic process is again experimentally demonstrated.

SUMMARY

An attempt was made to obtain further evidence in support of the conclusions reached by Dastur and Mehta (1935) on the importance of blue-violet rays in photosynthesis.

A carbon arc lamp, which is known to be rich in the blue-violet rays, together with an ordinary gas-filled electric lamp, were used and the carbohydrate contents of the leaves exposed to the two lights were determined in each case. Secondly, a beam of electric light is made richer, in the blue-violet rays by adding a beam of blue-violet light to the beam from a gas-filled electric lamp. This is termed 'mixed' light.

The results show that more carbohydrates are formed in leaves exposed to the arc lamp than those formed in leaves of the same plant exposed to the gas-filled electric lamp for the same period of time. Similarly, the carbohydrate contents of the leaves exposed to the 'mixed' light are higher than those of the leaves exposed to the electric lamp.

The carbohydrate contents of the leaves exposed to the carbon arc lamp are higher than those of the leaves exposed to the 'mixed' light.

The differences between the gas-filled electric and the arc lamps, between the gas-filled electric and 'mixed' light, and between the 'mixed' light and the arc lamp are statistically significant.

The importance of the blue-violet rays in the photosynthetic process is demonstrated quantitatively.

The Influence of Environment on the Growth and Metabolism of the Tomato Plant

II. The Relationship between Water Content and Assimilation

BY

R. MELVILLE, Ph.D.

(*From the Research Institute of Plant Physiology, Imperial College of Science and Technology and the Experimental and Research Station, Cheshunt, Herts.*)

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PRELIMINARY WORK

DURING the course of earlier experiments (Bolas and Melville, 1933) on the metabolism of seedling tomato plants some indication was obtained that assimilation might be influenced by the external conditions to which the plants had been exposed on the day preceding an experiment. It was therefore considered likely that an investigation of the assimilation rate of a group of plants on two consecutive days and of their translocation and respiration during the intervening night would throw some light on this effect of 'previous history'. The technique used earlier was employed with but slight modification.

The plants were placed in groups of four similar individuals by the 'paired plant' system of grading devised for the previous work (Bolas and Melville, 1933). One plant from each four was taken at 9.0 a.m. on the first day of an experiment, a second seven hours later, a third at 9.0 a.m. on the second day, and the fourth seven hours later. As a measure of the assimilation of the plants the percentage increase in their dry weight during the seven-hour period was employed. Significant results were obtained by using about

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twenty replicates. The assimilation for the first day was calculated from the difference between the means of the first and second samples, for the second day from the differences between the third and fourth samples. Translocation and respiration during the night were calculated from the differences between the means of the second and third samples.

It was foreseen that if this plan of experimentation was followed throughout the year, the seasonal variation in the length of the night would make comparable measures of translocation and respiration impossible; to avoid this difficulty the plants were darkened for the whole of the period between the second and third sampling. The results of the first experiment of this type were anomalous (Table I), the gain in dry weight during the second assimilation period being much less than for the first period, in spite of a higher light intensity. Temperatures on the two days did not differ significantly and, as far as was known, other environmental conditions were similar. The mean light intensity during the second assimilation period was approximately the optimal value indicated by the earlier work. There was one difference, however, in that the effective length of the intervening night had been increased by five hours by covering the plants between the second and third sampling times. The difference between the two assimilation values was shown by the application of Student's method (Fisher, 1928) to be highly significant.

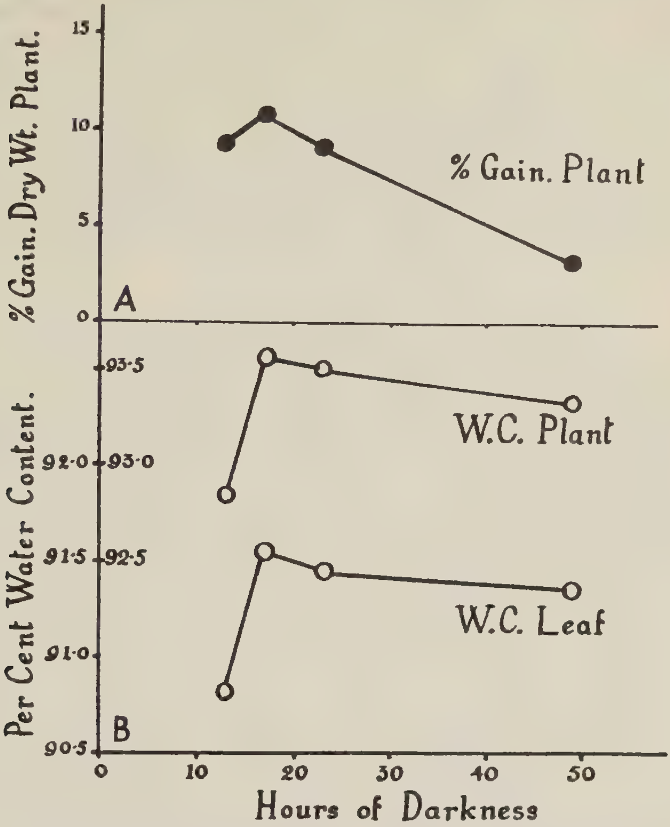
TABLE I

The Effect on the Plant of its Previous History

Date.	Time between sampling (hrs.).	Mean light (foot-candles).	Mean temp. (° C.).	Percentage gain in dry wt.		Initial water content of leaves.	Change in water content of leaves.
				Plant.	Leaves.	%	%
13.10.32	7	428	21·1	17·05	19·34	91·61	−1·48
	17	dark	16·1	−5·85	−10·15	90·13	+1·50
14.10.32	7	700	21·4	13·14	15·85	91·63	−1·04

In view of the results of this experiment it seemed probable that the length of the dark period preceding a period of assimilation was an important factor. Since an increase in the length of the dark period had been found to depress the assimilation rate it seemed likely, also, that a further increase might enhance this effect. With the object of testing this hypothesis, four groups of paired plants were subjected to periods of darkness of 49, 23, 17, and 13 hours, the last being the natural length of the night at the time of the experiment. The dark periods were arranged to end at the same time so that the four groups of plants should be exposed to the same conditions for assimilation.

Two experiments of the above type were made, but the size of the samples was limited by the number of plants available; each group consisted of six pairs of plants, a number too small to yield significant results for assimilation. Since the conditions of light and temperature during the two experiments did not differ very markedly, there is some justification for taking the average of the means as an indication of the effect on subsequent assimilation of increasing periods of darkness (Table II and Fig. 1). The results suggest that the effect of a slight increase in length of the dark period is to raise



FIGS. 1 A and 1 B. The effect on the plant of its previous history. Fig. 1 A. The percentage gain in dry weight of the whole plant for groups of plants previously exposed to darkness for different periods of time. Fig. 1 B. The percentage water contents of the same plants at the beginning of the assimilation period.

TABLE II

Assimilation after Long Periods of Darkness

Length of dark period (hrs.).	Percentage gain in dry weight in 7 hrs.			Percentage water content at end of dark period (2.11.32).	
	24.10.32.	2.11.32.	Mean.	Plant.	Leaf.
13	9·78	8·79	9·29	92·85	90·82
17	12·79	8·84	10·82	93·57	91·55
23	4·94	13·37	9·16	93·51	91·45
49	3·51	3·17	3·34	93·35	91·36
Mean light (ft.-c.)	172	158	—	—	—
Mean temp. (° C.)	18·9	17·8	—	—	—

the rate of the subsequent assimilation; this was confirmed by later work. Further increments result in the depression to an increasing extent of the assimilation.

In the second of the two experiments the fresh weights of the plants were obtained and the percentage water contents at the beginning of the assimilation period were calculated. There is a parallel between the water content and assimilation results in that the first increment in the length of the dark period resulted in a slight increase in the percentage water content, followed by small but continued decreases as the period of darkness was extended. Earlier work had shown that at any time the variations of percentage water content were small among plants of the same age and history.

PLAN OF THE EXPERIMENTS

In view of the result of altering the effective length of the night preceding a period of assimilation, the plan of experimentation was changed and the method outlined below was used throughout the later work.

Seedling tomato plants of the variety E.S. 1, grown under normal cultural conditions until they reached the eight-leaf stage, were used in the experiments. On the day preceding an experiment the plants were graded into pairs by the 'paired plant' system. Two groups were selected so that the individuals of any pair were in opposite groups, but otherwise at random. One group was subjected to six hours of darkness *in addition to* the natural night, while the other group served as the control. The length of the dark period preceding an experiment naturally varied for both groups according to the time of the year.

The 'long night' group was covered by a box consisting of a wooden framework covered by two layers of black fabric. This was placed in position six hours before sunset on the day preceding the experiment and removed at midnight by means of an electrical device. In this manner both groups of plants began the day under similar conditions and the length of the light period between dawn and the time of sampling, though varying at different times of the year, was the same for each treatment. The technique of sampling, drying, and weighing adopted was that previously described (Bolas and Melville, 1933).

THE MEASURE OF WATER CONTENT

The water content of a plant may be expressed in several ways. The simple method of stating the absolute water content by weight could not be used in this work as it was necessary to determine the relationship between assimilation and water content of plants of widely different fresh weights and to compare them at different times of the year. To make comparison possible the water content must be related to the dry weight. For this purpose it may be expressed either as a multiple of the dry weight or as a percentage of the fresh weight. The latter method is used in this paper.

Hitherto no easily measured characteristic of a plant has been known which would enable one to predict, with any degree of accuracy, assimilation under given conditions of light and temperature. The preliminary experiments suggested that percentage water content determined at the beginning of assimilation might be a characteristic of this type.

It is well known that the water content of a plant passes through a cycle of changes in the course of a day. If the percentage water content is determined at any particular stage in this cycle a relatively low variability is found among the individuals of a group of plants of the same age and previous history. As an example of these changes in a group of seedling tomato plants, the percentage water content of each plant in one representative experiment is given in Table III. The range of variation between the water contents is somewhat greater in the evening than in the morning. The mean changes between the percentage water contents of the pairs during the experiment were, for the leaf, 0.57, and for the whole plant, 0.87. When tested by Student's method, these changes were found to be highly significant on even such a small sample as ten plants; samples of not less than twenty plants were used in most of the experiments.

TABLE III

Water Content (%) Morning and Evening

	Whole plant.			Total leaf.		
	Morning (M.)	Evening (E.)	Difference	Morning (M.)	Evening (E.)	Difference
Pair No.	9.30 a.m.	4.30 p.m.	M.−E.	9.30 a.m.	4.30 p.m.	M.−E.
1	92·58	91·84	0·74	90·78	89·93	0·85
2	91·88	92·13	−0·25	90·36	90·08	0·28
3	92·30	91·48	0·82	90·47	89·16	1·31
4	92·13	91·84	0·29	90·57	89·67	0·90
5	92·63	91·71	0·92	91·00	89·71	1·29
6	92·25	91·49	0·76	90·33	89·61	0·72
7	92·20	92·01	0·19	89·97	90·06	−0·09
8	92·41	91·94	0·47	90·69	89·81	0·88
9	92·13	91·67	0·46	90·38	89·63	0·75
10	92·23	90·97	1·26	90·87	89·09	1·78
Means . .	92·274	91·708	0·566 (*P* = 0·01)	90·542	89·675	0·869 (*P* = 0·01)
Range of variation .	0·75	1·16		0·67	0·99	

In spite of the low variability of the percentage water content, it is possible to distinguish plants with relatively low or relatively high water content. With low water content the leaves are comparatively dark green and thick, and the stems develop a bluish anthocyan pigment, which may be present to a lesser extent in the leaves. Plants having a comparatively high percentage water content are paler green with thinner leaves, less chlorophyll, and are lacking in anthocyan pigment. The two types of plant merge into one another. They are distinguished by nurserymen as 'hard' and 'soft', respectively. In grading by the 'paired plant' system, differences of this type were taken into account in addition to plant height, stem thickness, and leaf area.

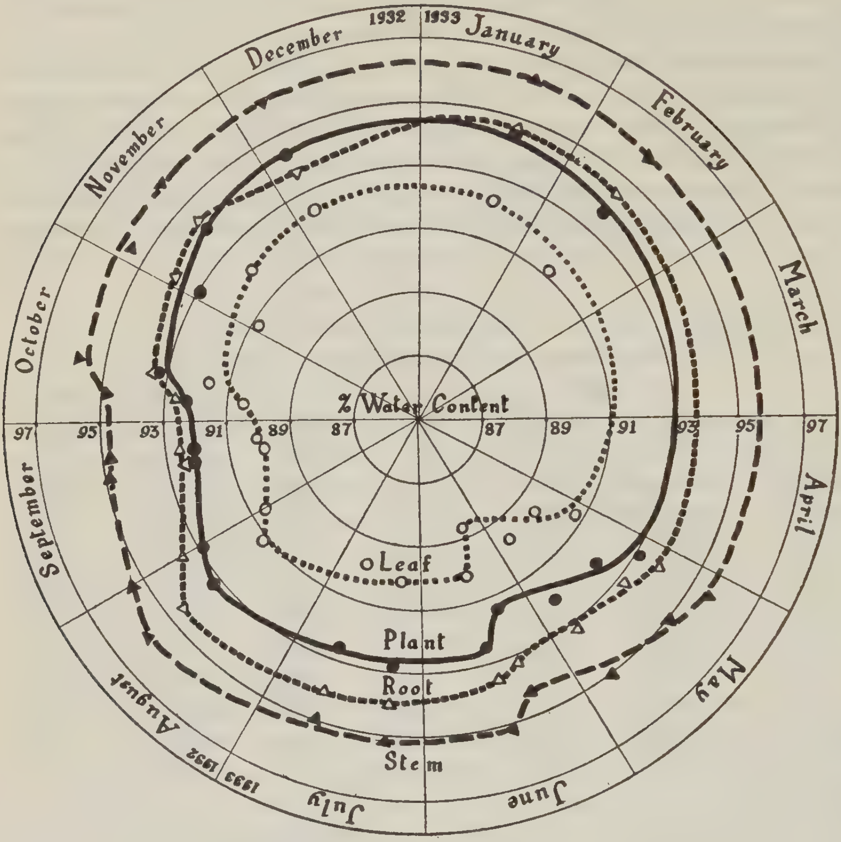


FIG. 2. The seasonal drift of percentage water content.

THE RELATIONSHIP BETWEEN PERCENTAGE WATER CONTENT AND EXTERNAL CONDITIONS

From the examination of the data of preliminary experiments evidence was obtained of marked seasonal changes in the percentage water-content of seedling tomato plants. Since the plants were in all cases at the same morphological stage of growth, it seemed evident that these changes were due to the effects of altering external conditions. The observations now extend over a full year and make a more detailed examination of the results possible.

The drift of percentage water content through the season is shown separately in Fig. 2 for the whole plant, the leaf, the stem, and the root. Water content was highest in the stems and lowest in the leaves of the seedlings used in this work. Both daily and seasonal fluctuations were greatest in the leaves, which respond more readily than other parts of the plants to changing environmental conditions. Fluctuations were smaller in the stem and least in the roots. It is probable that washing of the roots to remove the soil tended to reduce the size of the changes in them.

TABLE IV

Relationship between Morning Water Content (%) and Length of Night (Hrs.)

	Normal treatment.			Long-night treatment.		
	Length	Water content.		Length	Water content.	
Experiment.	of night.	Plant.	Leaf.	of night.	Plant.	Leaf.
22.8.32	8·8	93·30	91·20	—	—	—
31.8.32	8·8	92·95	90·58	—	—	—
19.9.32	10·3	92·16	89·91	—	—	—
21.9.32	11·0	92·13	90·07	—	—	—
7.10.32	11·7	92·27	90·54	—	—	—
13.10.32	11·9	93·38	91·61	—	—	—
2.11.32	12·4	92·89	90·82	17·0	93·57	91·55
14.11.32	13·4	93·98	92·03	22·0	94·12	92·11
5.12.32	14·5	94·27	92·36	22·0	94·54	92·68
19.1.33	15·5	94·52	92·28	21·0	94·78	92·53
4.5.33	10·1	93·15	90·75	19·0	93·48	91·31
11.5.33	9·5	92·16	89·64	18·0	92·72	90·60
25.5.33	8·7	92·15	89·75	18·0	92·81	90·71
9.6.33	8·2	91·49	88·78	14·0	92·23	89·51
14.6.33	7·7	92·56	90·18	13·0	92·99	90·77
7.7.33	7·5	92·92	90·20	13·0	93·56	91·10
20.7.33	8·0	92·55	89·84	13·0	93·07	90·45
26.7.33	16·0	93·87	91·84	22·0	94·06	92·01
Mean	10·8	92·93	90·69	17·7	93·49	91·28

Correlation (r) between Length of Night and Morning Water Content

	Normal treatment.		Long-night treatment	
	Plant	Leaf	Plant	Leaf
r	0·723	0·807	0·708	0·790
p	0·01	0·01	0.01	0·01

The percentage water content, as determined in the morning at a corresponding stage in the daily cycle of water-content changes, remained fairly constant in both summer and winter. The short intervening periods were marked by the occurrence of minimal water contents. During the summer the mean percentage water content of the plant was 92·86 and of the leaf 90·40. In the winter the mean values rose to 93·81 for plant and 91·82 for leaf.

In the course of the day the percentage water content of a plant falls. This can be accounted for by transpiration losses and to the effect of the accumulation of assimilates. The fall is made good during the night by the effects of growth, respiration, and enhanced water uptake under conditions of reduced transpiration. As the relative length of day and night alter through the season, an effect on the morning water content is to be expected. It can be seen from Table IV that a high and significant correlation exists between the morning water content and the length of the preceding night. The seasonal drift from a high percentage water content in the winter to a relatively low percentage water content in the summer may, therefore, be attributed to the complex effects of alterations in the relative lengths of day and night. This conclusion applies only to plants grown under glass-house conditions where artificial heating prevents a large seasonal fluctuation in temperature.

The seasonal effect of a decreasing length of day in winter includes a reduction of the amount of assimilate produced during a day on account of both the shorter assimilation period and the lower light intensity, thus the fall in the percentage water content due to the accumulation of assimilates is smaller. The longer night allows more time for recovery and for utilization of the assimilate. The respiration also remains relatively high in the glass-house during the night, and so tends to deplete further the available assimilate. These factors all favour the production of a high percentage water content in winter.

In late spring and in early autumn two periods occurred in which the percentage water content reached minimal values. The autumn minimum was reached when the day length was between thirteen and fourteen hours. The possibility that a critical length of day might be involved was considered, but in the following spring the minimum occurred when the day length was sixteen hours. Length of day alone is not therefore of much importance in determining the time of the minima.

Among the other external conditions considered was light intensity. The only record available for the whole of the period of the experiments was that of a Campbell-Stokes sunshine recorder, in which only bright sunlight is recorded. It is evident from the irregularity of the fluctuations in the amount of bright sunlight that light intensity was not responsible for the minima (Fig. 3).

An examination of the temperature records revealed a sharp rise in the mean night temperature at the time of the spring minimum of percentage water content. It was found that percentage water content tended to be low when night temperature was high. The correlation between percentage water content in the morning and the mean temperature during the preceding night is shown in Table V. The probability of the significance of the results was determined by Student's method, using the means of the individual experiments in order to reduce the labour of calculation. The significance of the results is therefore higher than represented. As night temperature is closely related to the temperature on the preceding day, similar correlations were obtained for percentage water content and the mean temperature on the preceding day. In the case of the leaf, the correlation was higher for the day temperature than for the night temperature. This must be regarded as a secondary result dependent on the accumulation of greater amounts of assimilates during days with high light intensities with which a high temperature is associated.

On the evidence of the correlations temperature is one of the factors controlling the percentage water content. From a comparison of the curves for percentage water content (Fig. 2) and mean night temperature (Fig. 3) the correlation appears to be closest during the months of May, June and July, the effect of temperature on water content having a lag of about a week. At the time of the spring minimum, the effect of a high night temperature was probably accentuated by the conditions affecting assimilation and water uptake. The day was approaching its maximum length, allowing a long time for the accumulation of assimilates and for high losses of water by transpiration.

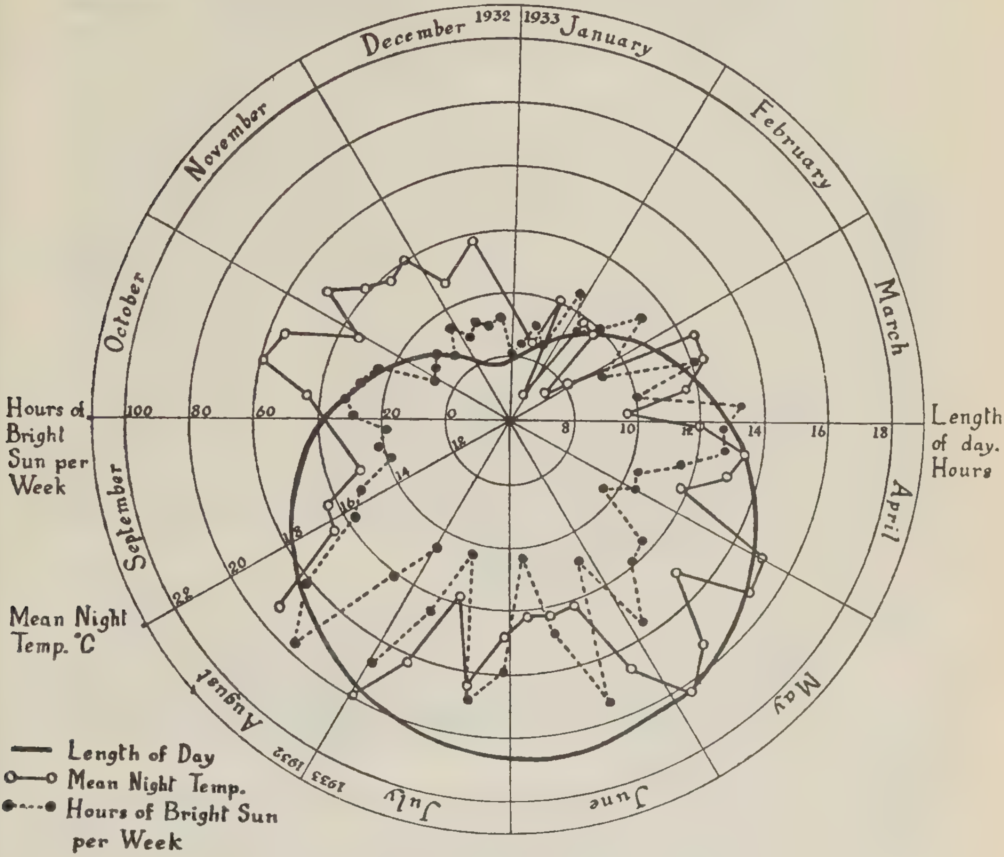


FIG. 3. Meteorological records for 1932–3.

TABLE V

Correlation between Morning Water Content (%) and Temperature

(*a*) Mean temperature of preceding night.

	Normal treatment.		Long-night treatment.	
	r.	p.	r.	p.
Plant	−0·614	0·02	−0·583	0·1−0·05
Leaf	−0·648	0·02—0·01	−0·582	0·1−0·05

(*b*) Mean temperature of preceding day.

Plant	−0·603	0·02
Leaf	−0·682	0·01

The short night allowed insufficient time for the utilization of the accumulated assimilates and for the recovery of normal water content under the more favourable conditions of transpiration. Such conditions would result in a lowering of the percentage water content, but would lead one to expect the minimum at the time of the shortest day. Actually, the minimum was two weeks earlier when the temperature at night was high on account of the glass-house being heated. Heating was discontinued at the beginning of June.

Though the correlation between mean night temperature and the percentage water content on the following morning is negative for the year as a whole, an examination of the graphs (Figs. 2 and 3) suggests that from September until the middle of December the correlation was positive. Accordingly the autumn minimum which occurred during this period was probably not due to the same causes as the spring minimum. It is known that the continued exposure of seedlings to a low temperature results in the 'hard' type of plant with a low percentage water content. September was a month of adverse weather with a continually falling temperature, which may account for the fall in the percentage water content. The glass-house heating was begun again in mid-September but did not at first stay the downward drift of temperature. From November onwards the high percentage water content may be attributed to the effects of the long night and the very low light intensities in the day. During this period temperature appears to have been relatively unimportant.

EFFECT OF THE LONG NIGHT ON THE PERCENTAGE WATER CONTENT

The percentage water content of the plants receiving the 'long night' treatment was always higher than that of the corresponding normal plants. The rise in the water content produced by the longer period of darkness is highly significant, as shown by Table VI in which the figures are based on the means of sixteen experiments.

The drift of the percentage water content in the long-night plants runs parallel to that of the normal plants over those portions of the year in which samples were taken (Fig. 4). The general features of the water-content drift and the correlation of percentage water content with both temperature and length of night receive strong support from this parallel series of experiments.

THE RELATIONSHIP BETWEEN PERCENTAGE WATER CONTENT AND ASSIMILATION

The only internal factor affecting assimilation rate that has hitherto been studied in detail appears to be chlorophyll content (Willstätter and Stoll, 1918). Water content has usually been considered of little significance. It is true that on account of the large amount of water in the cell, small changes of water content are unlikely to have a direct effect on the active mass of the substances concerned in the purely chemical phases of the assimilatory process. The permeability of the cell-membrane may perhaps be affected by quite small changes of water content. The rate of diffusion of carbon dioxide and other substances into the cell and of soluble assimilates out from it would in turn be changed. Water content may in this manner afford a rough measure of the internal factors other than chlorophyll content that control assimilation rate.

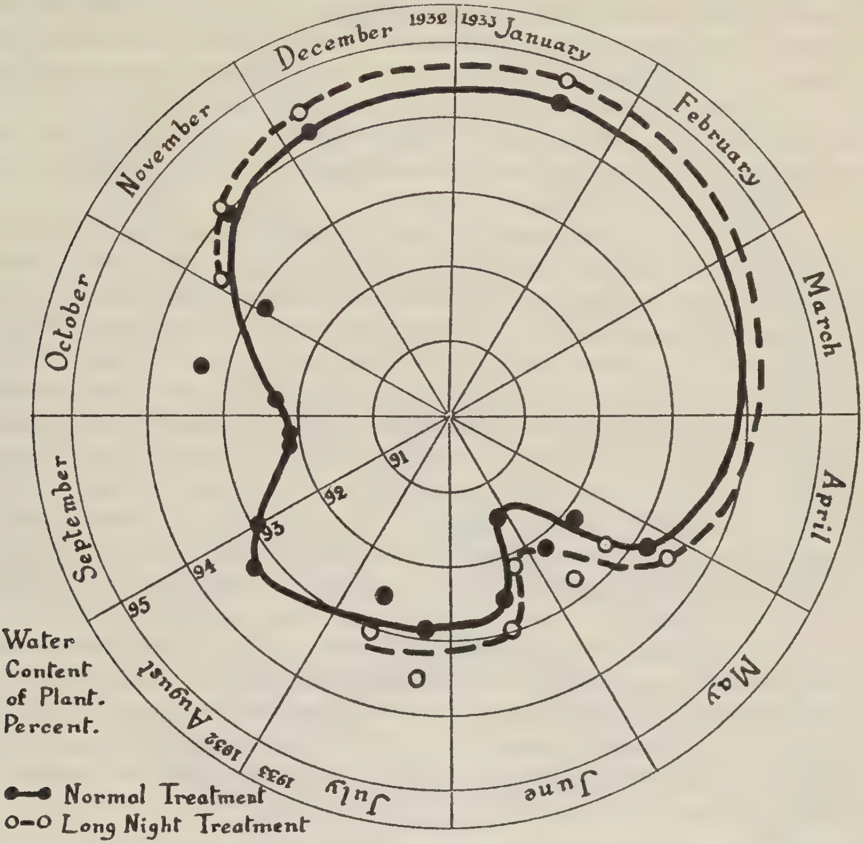


FIG. 4. Seasonal drift of percentage water content for normal night and 'long night' treatments.

TABLE VI

Rise in Water Content with Long-night Treatment

Water Content (%)

	Normal.	Long night.	Difference.	p.
Plant . .	92·86	93·32	0·46	0·01
Leaf . .	90·41	90·99	0·58	0·01

Evidence of the importance of water content in relation to the assimilation of a pure line wheat was given by Iljin in 1922. In his work the daily increase in dry weight was found to vary greatly with the dryness of the weather, and on particularly dry days losses in weight were recorded. The problem was here complicated by the respiration of the plants during the night. Experiments by Maximov in 1924 showed that a considerable loss of dry weight occurred during permanent wilting. This aspect of the subject was also studied by Brilliant (1924), who found a striking decline in assimilation rate of pieces of leaf allowed to wilt. In such a case closure of the stomata may be an important factor limiting assimilation rate by reducing the rate of diffusion of carbon dioxide into the leaf. Stomatal aperture is unlikely to have been a controlling factor on the assimilation of leaves on normal plants such as were used in the present work.

A linear relationship was found by Dastur (1925) between the water content of leaves of several plants (Abutilon, Spermannia, Cineraria) and their assimilation rate at constant temperature. In his work the water contents were determined at the end of the experiment. This method does not avoid the complications due to the accumulation of assimilate and to transpiration during the period in which assimilation is being measured. It is desirable that the water content should be determined at the beginning of the experiment. In a later paper Dastur and Desai (1933) studied the effect of water content and temperature on assimilation in air containing 5 per cent. of carbon dioxide, using the same technique. Their results are expressed as the ratio of the weight of carbon dioxide absorbed per square decimetre of leaf to the weight of water contained in the same area. Assuming a linear relationship to hold, this ratio should remain constant at a given temperature. A series of these ratios, determined at different temperatures, reflects the effect of temperature on assimilation rate. That this should be so is a confirmation of their hypothesis for the range of water contents they examined. Since the structure and thickness of leaves varies considerably at different seasons of the year, it is questionable how far such of their results as were obtained at different times are comparable.

SEASONAL DRIFT OF ASSIMILATION

Some evidence of a relationship between assimilation and the percentage water content determined at the beginning of the assimilation period was obtained from an examination of the seasonal drift of the percentage gain in dry weight of the plants. It may be seen from Figs. 4 and 5 that for the period from the beginning of May to the middle of October the curve for assimilation repeats the main features of that for percentage water content. In particular, the periods of minimum percentage water content coincide with the times of minimum assimilation. Whatever the cause of the low water content may be it is clear that at such times assimilation is closely correlated with it. The environmental conditions leading to the low percentage water content, therefore, were probably responsible for the low assimilation rate. In this sense the percentage water content may be said to epitomize the previous history of the plant.

During the winter and early spring, from mid-October until the end of April, the curves (Figs. 4 and 5) provide little evidence for a positive correlation between percentage water content and assimilation; several of the winter

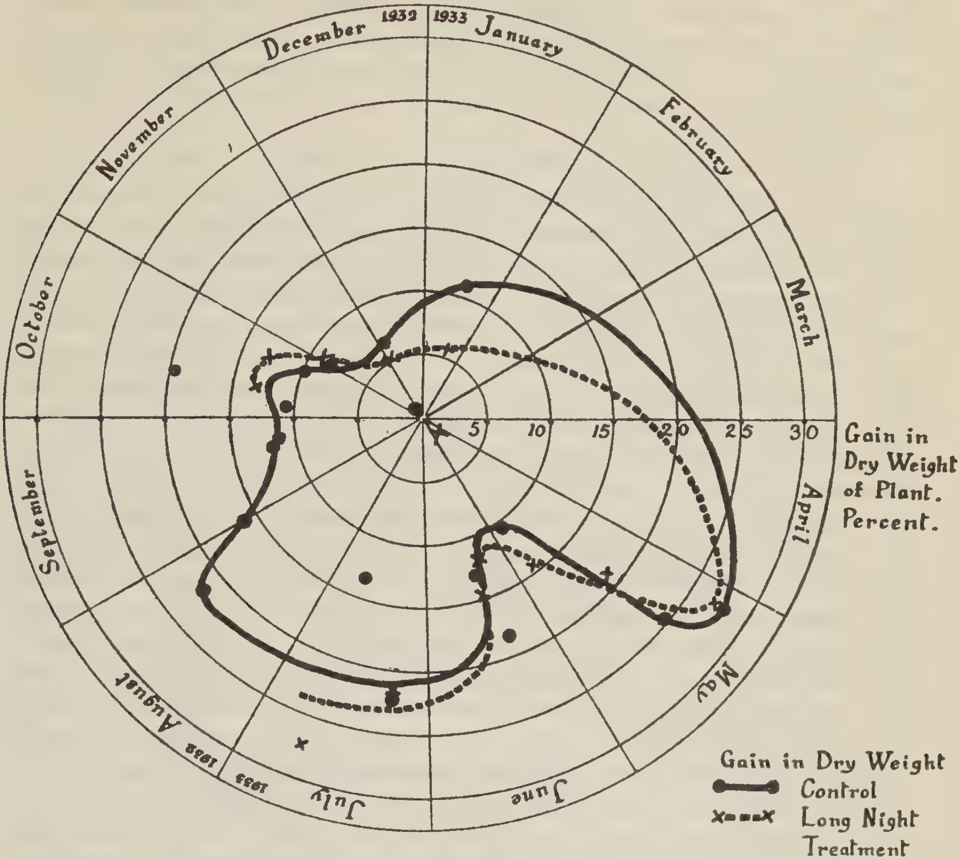


FIG. 5. Seasonal drift of assimilation for normal and long-night treatments. The percentage gain in dry weight of the whole plant during a seven-hour period is used as a measure of assimilation.

experiments did, in fact, suggest a negative correlation. It would seem that with the advent of winter some new environmental factors were introduced which upset completely the metabolism of the plants. The effect of the new conditions appeared to be cumulative, and recovery in the spring slow. Low light intensity and short length of day are probably concerned in this.

The lowest assimilation rates were found in November and were associated with relatively high percentage water contents. As a result of fogs the lowest light intensities also were encountered at this time. The minimum assimilation rate, for the same reason, did not coincide with the time of the shortest day as might otherwise have been expected.

The seasonal trend of the assimilation results was similar for plants exposed to both long-night and to normal treatment. The minimum in the spring coincided with the minimum of percentage water content, but no corresponding observations on 'long night' plants were made in September. In November assimilation fell below that of the controls and remained lower in December and January, though in all cases the percentage water content was higher. This may be attributed to the adverse environmental conditions referred to above, enhanced by the effect of the extended night.

Correlation between Water Content, Light, and Assimilation

When correlation diagrams were plotted for percentage water content of the leaves, determined in the morning, and the percentage gain in dry weight of the whole plant during the subsequent seven hours, there was no evidence of a close relationship. It was obvious that by taking the results of the whole season together correlations existing in the summer were obscured by divergent observations made under winter conditions. Similarly, no close correlation was indicated between either light or temperature and assimilation rate for the results of the entire season. This clearly was due to the interaction of other factors, at least in the case of light and temperature.

If a pair of factors is considered, it is possible to study the effect of each on assimilation rate by the use of a three-dimensional diagram. Such a diagram could be constructed for percentage water content and light, and for percentage water content and temperature. In nature, and to a less extent under glass, temperature depends mainly on meteorological conditions and tends to follow the fluctuations of light intensity. As light causes larger fluctuations in assimilation rate than does temperature, the effect of temperature tends to be masked by that of light when these factors are not separated. In consequence of this and the limited data it was not possible to construct a solid diagram for percentage water content, temperature, and assimilation.

The procedure adopted for the construction of the three-dimensional diagram was as follows. Suitable scales for percentage water content of leaf and for mean light intensity in foot-candles were marked off at right angles to one another on a rectangular board. The percentage gain in dry weight of the whole plant, as a measure of assimilation, was plotted on a vertical scale. Individual assimilation results were represented by vertical wires of suitable length placed in the appropriate positions. Two sets of smoothed curves parallel respectively to the water content and light axes were constructed in thick copper wire. The two sets of curves were soldered at their intersections, and the surface thus formed was contoured with thin copper wire at levels representing 5 per cent. increments of gain in dry weight. By making the surface in this way it was possible to take all the data into account when estimating the form and level of each constituent curve. Portions of some of the curves were obtained by extrapolation.

The relationship between water content and assimilation at four different light intensities is shown by the curves in Fig. 6. There is a marked rise in assimilation as the initial water content increases from 89 to about 91·5 per cent. The maximum assimilation was attained at different percentage water contents for each range of light intensity, suggesting some interaction between these factors. With higher water contents than 91·5 per cent. there was a rapid decline in assimilation. The form of the surface over this descending slope is speculative and its nature is discussed below.

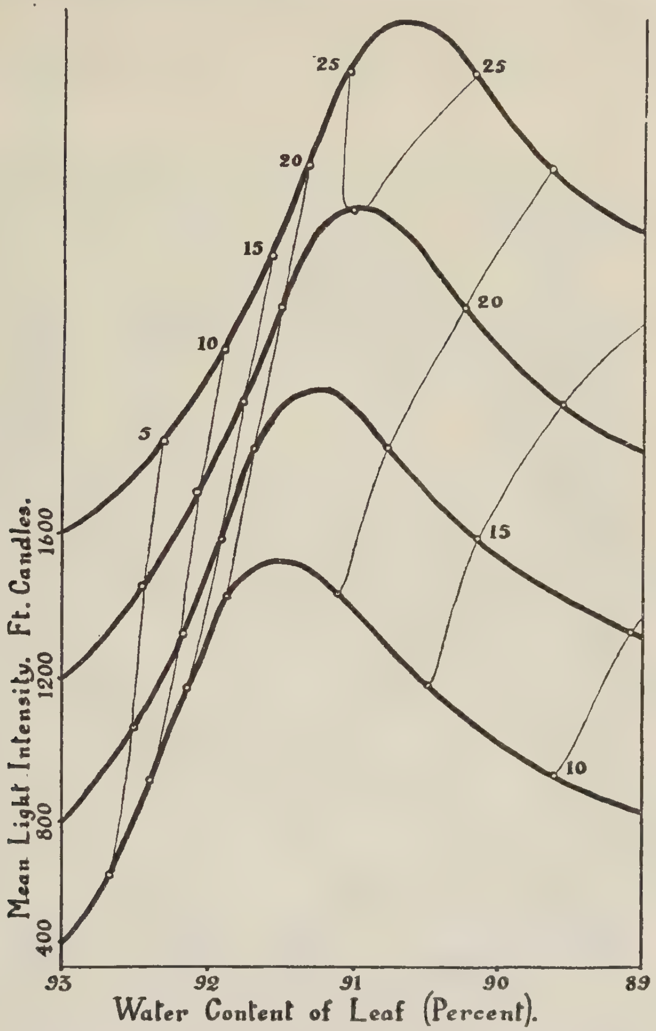


FIG. 6. Assimilation and percentage water content. Four curves from the solid model showing the relationship between assimilation and percentage water content for four light intensities. The curves are separated by equal arbitrary distances and 5 per cent. levels of assimilation are marked, and corresponding points joined to form contours.

The four curves in Fig. 7 show the variation of assimilation with light intensity for initial water contents of 89, 90, 91, and 92 per cent. There is in each case a rapid increase in assimilation as the mean light intensity rises from zero to about 200 foot-candles. Over this range, light intensity is apparently the controlling factor. Above 200 foot-candles, assimilation increases but slowly with rise in light intensity up to a mean of 1,600 foot-candles. The curve for 92 per cent. water content falls after reaching a maximum, since it passes over the oblique crest of the surface.

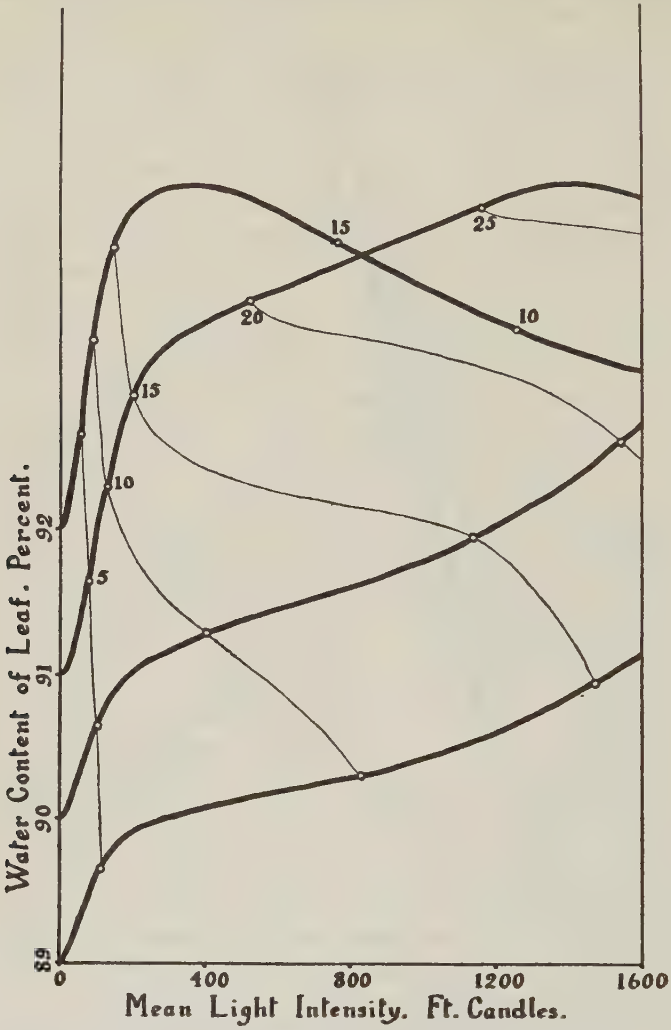


Fig. 7. Assimilation and light intensity. Curves from the solid model showing the relationship between assimilation and light intensity for four initial percentage water contents. The curves are separated by arbitrary intervals and the contours formed as in Fig. 6.

Fig. 8 is a contoured plan of the surface. This expresses all the relationships referred to above.

EFFECT OF THE LONG-NIGHT TREATMENT ON THE CORRELATIONS

The general conclusions drawn from the normal series of experiments gain strong confirmation from an examination of the results of the parallel series in which the plants were given six hours of darkness in addition to the hours of night normal for the time of the year. The solid diagram based on these data is of similar form, but is found to occupy a position farther along the water-content axis in the direction of higher water content. It projects beyond the normal surface on the high water-content side and falls below it on the low water-content side.

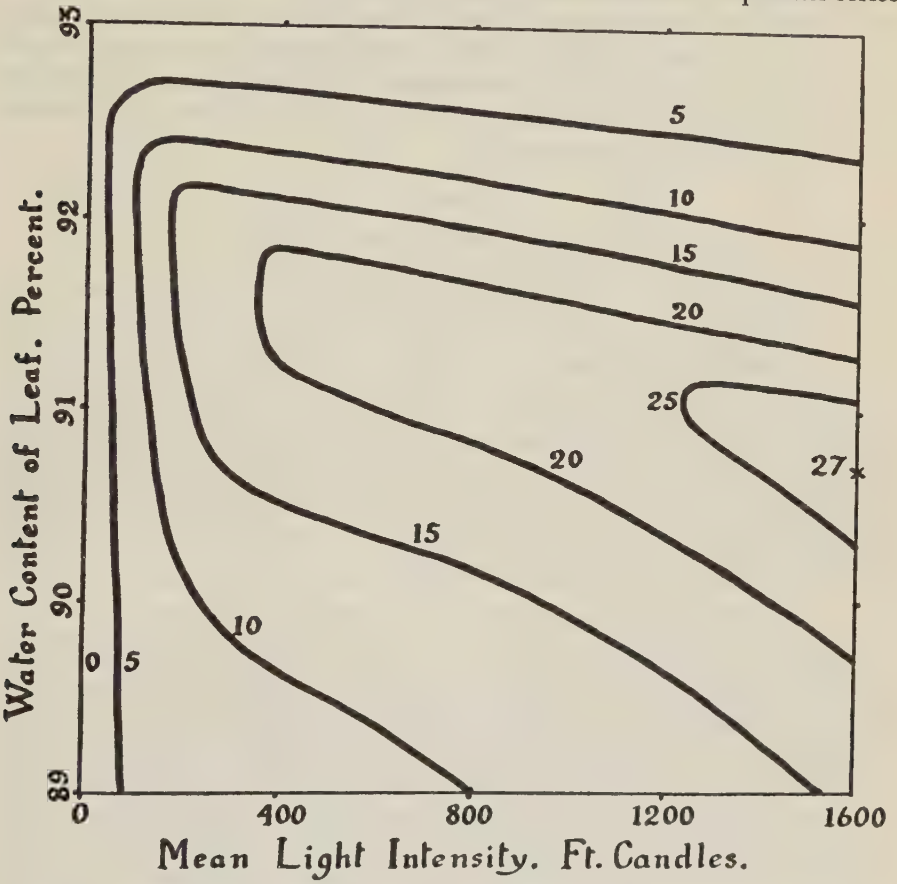


FIG. 8. Contour plan of the solid model. The numbering of the contours indicates the percentage gain in dry weight of the whole plants.

If the rise in percentage water content and the associated increase in assimilation were the only effects of the long-night treatment the two surfaces should have been coincident. The shifting of the surface indicates that other physiological processes are modified, and among them translocation during the assimilation period is probably one of the more important. By lengthening the night a longer time is available for the leaf to be cleared of assimilates from the preceding day, for absorption of water, and for growth. The effect of this should be to decrease the initial translocation gradient from the leaves to the stem and root at the beginning of the following assimilation period. Compared with normal plants, a relatively smaller increase in the dry weight of the stem and root might then be expected. The proportional distribution of the gain in the dry weight of the plant, shown in Table VII, provides some support for the above argument.

TABLE VII

Distribution of the Gain in Dry Weight

	Percentage of Total Gain.		
	Leaf.	Stem.	Root.
Normal treatment	70·28	21·90	7·82
Long-night treatment	72·37	21·56	6·12

For a particular percentage water content a smaller relative increase in the weight of the stem and root causes a smaller percentage increase in the dry weight of the plant as a whole when compared with normal plants. This effect would be expressed in a solid model by a lowering of the surface. It therefore seems probable that a lower rate of translocation was an important factor in causing the shifting of the surface.

EVIDENCE OF A PATHOLOGICAL WATER CONTENT

The rapid decline in assimilation when the water content rises above 91·5 per cent. has already been alluded to. Such high water contents occur in glass-house practice only in the winter months, and then only during periods of foggy weather. The data on this point are in consequence rather scanty. In the experiments in which the abnormally high water contents occurred, the long-night treatment raised the water content still farther, but the assimilation was depressed. The usual effect of the long night on assimilation therefore was reversed. It is probable that a pathological state is set up in plants that have been subjected to such conditions, and autolysis of the protoplasm may begin.

It seemed desirable to obtain evidence of pathological water content during the summer months. With this object in view a group of plants was subjected to an eight-hours' day during the whole period of their development from the time the cotyledons were expanded. Control plants reached the eight-leaf stage used in this work in five weeks, but the short-day plants took eight weeks. They had smaller leaves, thinner stems, and less chlorophyll than normal summer plants, and thus resembled winter plants in these characters. Half of the group was given an additional six hours of darkness on the night preceding the experiment, the two sets receiving twenty-two hours and sixteen hours, respectively. A comparatively small rise in the percentage water content resulted from the extra six hours of darkness and was followed by a slightly greater assimilation rate (Table VIII). The difference, however, was not significant.

TABLE VIII

Assimilation of Short-day Plants

Date 26.7.33. Seven hours' assimilation; mean light 1309 ft-c.; mean temp. 19·1 C.°

Length of night.	Gain in dry weight (%).		Morning water content (%).	
	Plant.	Leaf.	Plant.	Leaf.
16 hours	12·60	17·40	93·87	91·84
22 ,,	13·18	20·52	94·06	92·01

The gain in dry weight of the 'Short-day' plants under both treatments was approximately half that of normal plants in an earlier experiment in which the light intensity was similar but the percentage water content lower. Differences of chlorophyll content probably contributed to the lower assimilation rate of the short-day plants. The result was nevertheless of the type predicted from the winter experiments.

Some early experiments are favourable to the view that a high percentage water content is indicative of a pathological condition of the plant. With successive increments in the length of the period of darkness preceding an experiment both percentage water content and assimilation rate rose at first and then later continued to fall (Fig. 1). It would seem, therefore, that not only should there be a descending slope on the high water-content side of the solid model, but that this part of the surface should be re-entrant. The leaves of some similar winter plants were tested for starch by decolorization in 70 per cent. alcohol and the application of iodine solution. In plants that had been in darkness for twenty-four hours, only traces of starch were found. Further tests on comparable leaves showed that the normal amount of starch was not present after a period of assimilation until at least three days had elapsed under normal conditions. It seems probable, therefore, that pathological changes in the plant had accompanied the high water content in this case.

THE GOODNESS OF FIT OF THE SURFACE OF THE SOLID MODEL

The difference between the experimental values for assimilation and those indicated by measurements on the surface of the solid model give some indication of the closeness of fit of the surface to the results. It is evident that the individual differences and the mean arithmetic difference should both be small. From Table IX it is seen that the agreement is reasonably good for the surface relating to the normal treatment, for which the mean arithmetic difference is 2·6. The surface for the long-night treatment gives a mean difference of 4·1, indicating a greater variability of the results.

The results of a few later experiments are available to test the value of the surface for predicting assimilation (Table X). The error of prediction was low in all but two of these, in spite of the fact that most of the experiments were made in October and November when weather conditions are unfavourable. On the whole, these experiments indicate that the surface is a fairly

TABLE IX

Experimental Results and Goodness of Fit of the Surface of the Solid Model

			Normal treatment.			Long-night treatment.		
			Percentage gain in dry weight of plant.					
Date.	Mean light. foot-candles.	Mean temp., ° C.	Experimental.	From solid.	Difference.	Experimental.	From solid.	Difference.
22.8.32	548	20·2	22·0	20·6	−1·4	—	—	—
31.8.32	779	23·5	16·0	17·9	+1·9	—	—	—
19.9.32	271	15·0	12·0	10·7	−1·3	—	—	—
21.9.32	812	20·5	11·2	14·4	+3·2	—	—	—
7.10.32	193	21·0	10·8	11·1	+0·3	—	—	—
13.10.32	428	21·5	19·7	20·9	+1·2	—	—	—
2.11.32	158	18·5	8·4	11·7	+3·3	8·8	11·4	+2·6
14.11.32	45	17·0	0·9	5·0	+4·1	−1·6	2·7	+4·3
5.12.32	97	18·5	6·7	10·0	+3·3	5·3	5·3	0·0
19.1.33	57	17·5	10·9	6·5	−4·4	5·8	5·2	−0·6
4.5.33	1,184	25·5	28·0	23·7	−4·3	27·1	24·2	−2·9
11.5.33	878	21·5	19·7	12·7	−7·0	18·9	17·4	−1·5
25.5.33	1,056	20·2	10·8	14·6	+3·8	14·4	20·0	+5·6
9.6.33	1,630	21·9	18·3	15·3	−3·0	11·8	16·6	+4·8
14.6.33	804	15·4	9·8	14·1	+4·3	12·5	19·0	+6·5
14.6.33	1,049	19·6	13·1	17·1	+4·0	14·6	20·4	+5·8
14.6.33	616	22·6	10·1	9·2	−0·9	14·6	9·6	−5·0
7.7.33	1,441	23·3	22·2	21·8	−0·4	21·7	25·1	+3·4
20.7.33	393	16·7	7·4	9·9	+2·5	9·1	11·3	+2·2
20.7.33	579	22·0	12·9	12·2	−0·7	27·4	13·7	−13·7
20.7.33	631	24·9	11·9	10·2	−1·7	16·3	9·5	−6·8
26.7.33	1,309	19·1	12·6	12·6	0·0	13·2	13·2	0·0
Algebraic total					+6·8			+4·7
Arithmetic total					57·0			65·7
Mean arithmetic difference					2·6			4·1

TABLE X

Predicted and Observed Gain in Dry Weight

				Gain in dry weight of plant (%).		
Date.	Mean temp. (° C.).	Mean light (ft.-c.).	Morning water content of leaf (%).	Predicted.	Actual.	Difference.
27.9.33	22·4	146	91·51	13·4	14·2	−0·8
4.10.33	23·8	823	90·96	20·9	17·7	+3·2
11.10.33	23·3	517	91·51	21·7	25·7	−4·0
10.11.33	17·7	191	91·19	14·9	4·5	+10·4
14.11.33	20·2	199	91·84	18·1	16·9	+1·2
16.11.33	19·2	125	91·57	12·4	1·0	+11·4
29.11.33	18·1	82	91·74	10·9	7·0	+3·9

good representation of the interaction of light and percentage water content on the assimilation of seedling tomato plants of the type and stage of growth used in this work.

SUMMARY

The percentage water content of seedling tomato plants of the same age and history varies little from plant to plant. Differences of the order of 0·5 per cent. are significant with samples of ten.

A significant rise in the percentage water content is brought about by exposure to six hours of darkness in addition to the normal night.

A seasonal drift occurs in the percentage water content of seedling tomato plants grown in a glass-house.

A negative correlation of 0·6 is found between mean night temperature and the percentage water content on the following morning.

A positive correlation of 0·8 is found between the length of the night and the percentage water content on the following morning.

A three-dimensional correlation diagram for percentage water content, light intensity, and gain in dry weight is constructed. The chief points emerging are: (*a*) the gain in dry weight increases to a maximum as water content increases; (*b*) further increase of water content results in a rapid decline in the rate of gain in dry weight; (*c*) the optimal percentage water content is dependent on light.

The surface of the solid model is examined for goodness of fit and is found to be satisfactory.

The percentage water content of the leaves at the beginning of a period of assimilation gives an indication of the effect of internal factors on the subsequent assimilation. The internal factors are dependent on the previous history of the plant.

In this paper one aspect of the growth relationships of the tomato plant is discussed. The experiments which provided the data were made possible by co-operation with my colleagues Mr. B. D. Bolas and Mr. I. W. Selman, for only by close teamwork could the numerous observations and manipulations be made in the brief period of an experiment. Grateful acknowledgement is made of the unfailing encouragement of Professor V. H. Blackman and of Dr. W. F. Bewley, Director of the Experimental and Research Station, Cheshunt, Herts., and thanks are due to Dr. F. G. Gregory for his interest and for advice on the mathematical treatment of results.

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The Effect of Alternate Periods of Light and Darkness of Short Duration on the Growth of the Cucumber

BY

G. B. PORTSMOUTH

(*From the Research Institute of Plant Physiology, Imperial College of Science and Technology*)

With six Figures in the Text

1. INTRODUCTION

THE experiments of Garner and Allard (1931) on the effect of alternating equal periods of light and darkness, varying in duration from five seconds to twelve hours, showed that the length of the intermittent period had a profound effect on the vegetative development of the plant and a much smaller effect on the flowering. They further noted a well-marked maximum effect in the neighbourhood of one-minute alternations, vegetative development here showing a very definite minimum. No explanation of this phenomenon was put forward by these authors. It has, however, been shown in an investigation by Gregory and Pearse (1937) that short alternating periods of light and darkness lead to partial closure of the stomata in *Pelargonium zonale*, again showing a maximal effect in the neighbourhood of one minute. As soon as this observation had been established it appeared desirable to investigate, on a quantitative basis, the effect of alternating periods of light and darkness on vegetative growth, in order by growth analysis to separate if possible the various factors concerned. This paper deals with an investigation on the growth of the cucumber (*Cucumis sativus*) under controlled conditions of light, temperature, and humidity.

2. EXPERIMENTAL PROCEDURE

(a) *Material.*

Before finally deciding to work with *Cucumis sativus* two preliminary trials, with a large variety of plants,[1] including many of those used by Garner and Allard, were carried out, using one-minute alternations of light and darkness, with twelve-hour periods of light as a control.

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[1] The plants grown in these trials were: *Alyssum* var., *Anagallis arvensis*, *Androsace filiformis*, *Aster* var., *Barbarea praecox*, *Beta vulgaris*, *Brassica campestris* var., *Cochlearia danica*, *Cordyline australis*, *Cucumis Melo* and *sativus*, *Cucurbito Pepo*, *Galium aparine*, *Glycine* vars. (2), *Helianthus annuus*, *Hordeum vulgare* vars. (2), *Ipomaea* var., *Nicotiana* var., *Papaver Rhoeas*, *Phaseolus multiflorus* and *vulgaris*, *Phlox* var., *Pisum sativum*, *Raphanus sativus*, *Ricinus communis*, *Rudbeckia* var., *Salvia coccinea*, *Silene armeria* and *rubella*, *Spergula arvensis*, *Tropaeolum* var., *Vicia Faba*, *Viola tricolor* var., *Zea Mays*, and *Zinnia* var.

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Considerable differential responses were shown by the majority of the species tested, the most marked differences under these conditions being exhibited by the French bean (*Phaseolus vulgaris*) and the cucumber (*Cucumis sativus*). As, however, the methods of study regarding the growth of the cucumber under controlled conditions had already been worked out in this Institute by Gregory (1921, 1928), it was decided to employ this plant in the present investigation.

(b) *Experimental chamber.*

This was the small greenhouse, measuring 10 ft. × 7 ft. × 8 ft., originally used by Gregory (1921) and situated within the greenhouse laboratory of the Institute. The outside of this house was covered with uralite sheeting, which served both to convert it into a darkroom and at the same time to give some measure of heat insulation. Temperature was controlled to within a degree Centigrade by a sensitive mercury thermo-regulator actuating two tubular electric heaters fixed to the walls. Just below the roof was installed a grid of cooling pipes through which brine could be circulated from the tank of a large refrigerator, the electric motor operating the circulating pump being also controlled by the thermo-regulator. The whole cooling system could, however, when not required, be put out of operation independently of the heaters.

Humidity was maintained at the desired high level by blowing air through a circular sheet of spray produced by two opposed water-jets mounted on the same axis. The spray and fan were fixed low down on the end wall and, in conjunction with another fan fixed above the door at the opposite end of the chamber, produced satisfactory circulation of the air.

Illumination was provided by two 1,000-watt gas-filled lamps suspended above the table supporting the plants, the vertical distance of the filaments above the soil-level being 80 cm. A screen of running water was fixed below the lamps to cut off heat radiation. During experiments with alternating light these lamps were operated by a heavy-duty magnetic relay which was controlled for long-period alternations by a Venner time switch, and by a motor-driven commutator for short-period alternations.

(c) *Cultural details.*

The same variety of cucumber was used as for previous experiments in this Institute (*Cucumis sativus* var. Butcher's Disease Resister). The seeds were graded and only those of the weight 37–41 mg. were used. These were sown singly in small earthenware pots, 3½ in. in diameter, in a sterilized mixture of 3 parts loam to 1 part well-rotted stable manure of sufficient volume to half fill the pot. Enough seeds were sown to supply 80 pots of uniform plants for each experiment.

After sowing, the pots were placed in the experimental chamber on a table, 4 ft. square, carrying eight wooden battens, 2 ft. × 1 ft. These battens were arranged as shown in Fig. 1 and with their respective pots were moved into a new position each day in order to compensate for any differences in conditions: one such change is shown in the diagram.

The pots were kept covered during germination and were all exposed to light at the same time. Four days after the plants appeared, an extra measure of soil was added to fill the pot and so allow of adventitious root formation from the hypocotyl. Water, drawn from a storage tank kept inside the chamber and thus at air temperature, was given once daily.

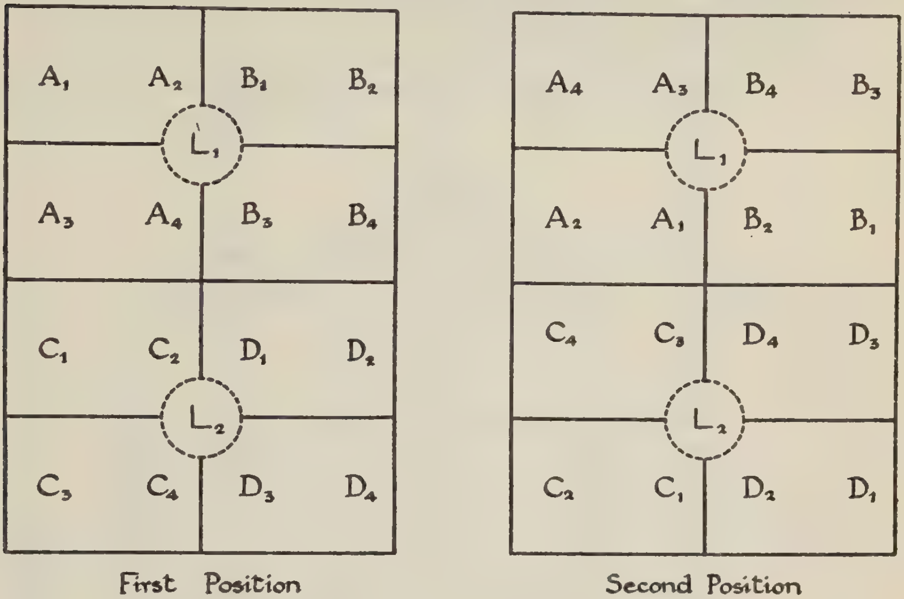

FIG. 1. For explanation see text.

(d) *Growth observations.*

In order that as much information as possible might be obtained from each experiment a comprehensive system of growth measurements and plant sampling was carried out. Leaf-area measurements, according to the method of Gregory[1], were taken at the same hour every second day on plants selected at random, and measurements of internode length recorded at the same time; while every fourth day a random sample of six plants was removed from the experimental chamber. On the plants sampled leaf tracings were taken first and the plants then separated into leaves, stems, and roots. Four plants were used for fresh and dry weight determinations; the other two were placed in boiling alcohol and kept for sugar analysis. Measurements and weights of all plants were recorded individually.

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[1] Measurements are taken of the length and width of the leaf between the various lateral points, together with the angles subtended at the base by lines joining these points. The area can then readily be worked out by the use of appropriate formulae. Reference should be made to the original paper (Gregory, 1921) for full details of the method.

(e) *Details of experiments.*

Three experiments in all were carried out, differing only in the type of illumination supplied, as stated below:

Experiment 1. Twelve-hour alternations of light and darkness.
Experiment 2. One-minute alternations of light and darkness.
Experiment 3. Continuous light.[1]

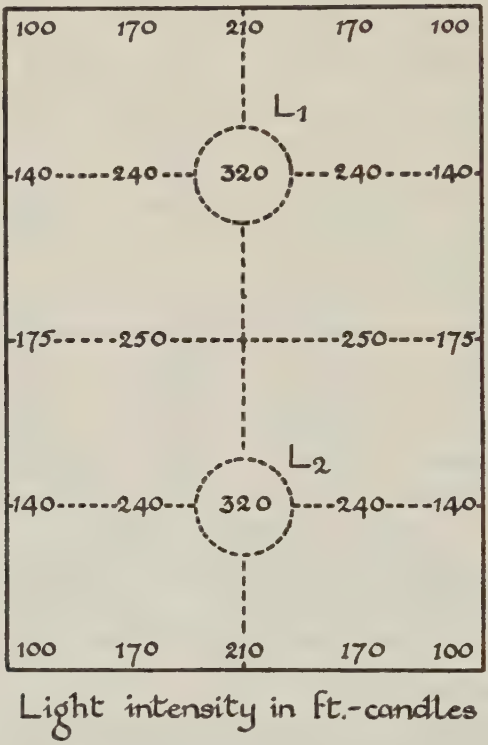

FIG. 2. Light intensities in foot-candles at various positions under the lamps.

The following details regarding the other controlled factors apply to all three experiments: *Temperature*, 25° C., maximum variation less than one degree; continuously recorded by thermograph. *Relative Humidity*, 90 per cent. maintained between the limits of 85–95 per cent.; continuously recorded by hair hygrograph and checked at intervals with an Assman psychrometer. *Light Intensity*, regular readings at pot-level were carried out periodically with both a Holophane Lumeter and a Weston Photometer and no fall in intensity was observed during the course of any one experiment. Fresh lamps were fitted at the beginning of each experiment. A diagrammatic schema of the distribution of light intensity in foot-candles at pot-level is given in Fig. 2.

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[1] In the absence of the author this experiment was carried out by Dr. Sheng Han Shih.

3. EXPERIMENTAL RESULTS

(a) *Increase in area of the leaf surface.*

In the present series of experiments the effects of short periods of intermittent light appear most markedly in the increase in leaf surface, many of the other effects being directly related to the resulting differences in effective total leaf area. In Fig. 3 are shown the curves of increase of total leaf area per plant with time in the three sets of experiments, the numerical data of which are presented in Table I.

TABLE I

Total Leaf Area

(Sq. cm. per plant.)

	Experiment 1. 12-hr. alternation.			Experiment 2. 1-min. alternation.			Experiment 3. Continuous light.		
Days.	A.	B.	C.	A.	B.	C.	A.	B.	C.
2	7·63	7·16	9·8	5·80	5·94	1·80	6·19	6·64	6·60
4	11·06	—	13·5	7·65	—	5·30	10·58	—	9·68
6	18·19	18·09	18·7	12·15	12·22	9·98	14·54	15·61	14·21
8	24·71	—	25·8	16·82	—	15·64	19·34	—	20·84
10	35·64	36·64	35·7	20·63	20·51	22·16	32·00	32·53	30·58
12	50·96	—	49·3	28·52	—	29·45	45·12	—	44·87
14	70·38	71·77	68·2	36·82	36·24	37·46	67·38	69·83	65·84
16	96·54	—	94·4	46·44	—	46·14	100·88	—	96·60
18	136·13	133·87	130·6	57·74	56·00	55·46	134·14	137·53	141·74
20	(175·86)	—	180·3	64·95	—	65·37	—	—	—
22	(242·58)	(238·71)	249·5	(76·30)	(73·80)	75·86	—	—	—

Values in column A are calculated from measurements made according to the method of Gregory, while values in column B are direct measurements with a planimeter. Column C is calculated from the equation of the curve of closest fit, only the region below the dotted line being used for this calculation. Figures in brackets include cotyledon area, although these had then withered.

The values in the first column for each series are those obtained by Gregory's method, while the second column gives those obtained by direct measurement of the leaf tracings with a planimeter. The extremely good agreement of these two sets of figures is striking and furnishes a corroboration of the accuracy of the former method.

Both the curves obtained in experiment 1 (12-hour alternations) and in experiment 3 (continuous light) may be represented by functions of the type $A = ae^{rt}$, the logarithmic forms of the calculated equations of the curves of closest fit being:

12-hr. alternation: expt. 1. $\log_{10} A = 0{\cdot}0704t + 0{\cdot}8486.$

Continuous light: expt. 3. $\log_{10} A = 0{\cdot}08325t + 0{\cdot}653.$

In experiment 2 (1-minute alternations), however, the curve is definitely not exponential but may be represented by a function of the form $A = at^r$, the logarithmic form of the calculated equation of the curve of closest fit being:

1-min. alternation: expt. 2. $\log_{10} A = 1{\cdot}561 \log_{10} t - 0{\cdot}2155.$

Thus short periods of light and darkness have not only very much reduced the rate of increase in leaf surface but have caused a departure from the normal exponential type of growth. There is very little difference between experiments 1 and 3, except for the somewhat higher growth rate exhibited in the latter: growth, however, did not begin so well under continuous light, probably owing to some delay in the expansion of the cotyledons.

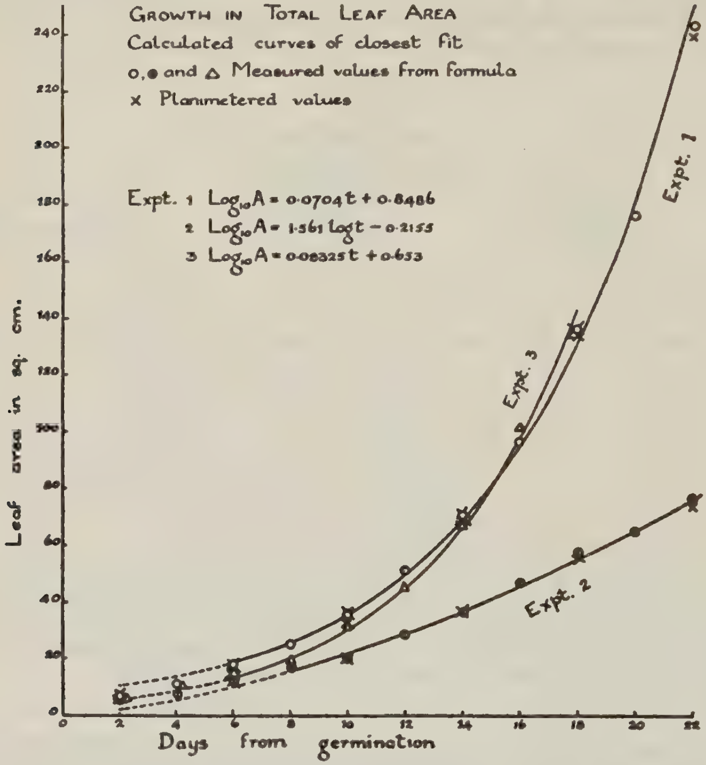

FIG. 3. Growth in total leaf area of plants; average values for random samples of six plants. Expt. 1, twelve-hour alternation; expt. 2, one-minute alternation; expt. 3, continuous light.

(b) *Increase in stem length.*

Again both the curves obtained in experiments 1 and 3 are quite regular and approximate to the normal exponential type, the logarithmic forms of the equations of the curves of closest fit being:

12-hr. alternation: expt. 1. $\log_{10} L = 0{\cdot}0308t + 0{\cdot}8701$.

Continuous light: expt. 3. $\log_{10} L = 0{\cdot}031t + 0{\cdot}758$.

These two curves are almost exactly parallel, as can be seen in Fig. 4; but, as in the case of the curves of increase in leaf surface, growth under continuous light begins at a lower level than under twelve-hour alternations of light and darkness. This effect is entirely due to decreased extension of the hypocotyl and is no doubt intimately associated with the factor causing delay in expansion of the cotyledons previously mentioned. The curve obtained in experiment 2 is strikingly dissimilar in appearance from the other two, and

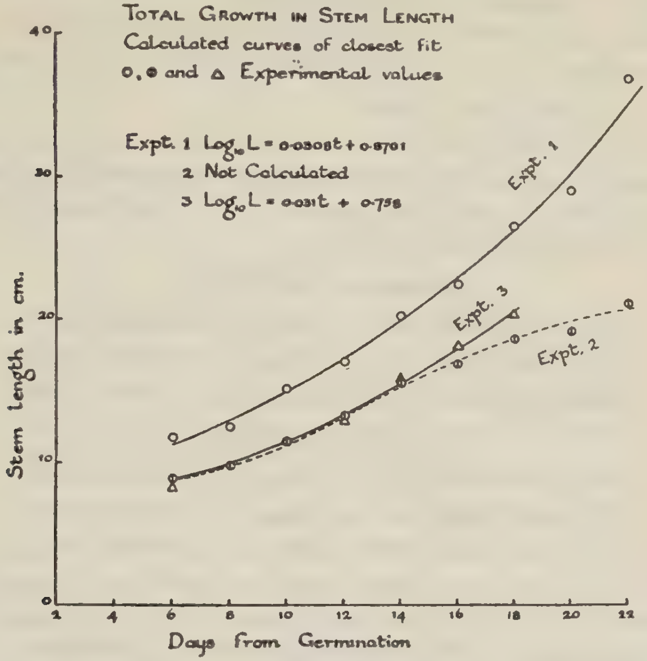

FIG. 4. Growth in total length of stem in cm.; average values for random samples of six plants. Expt. 1, twelve-hour alternation; expt. 2, one-minute alternation; expt. 3, continuous light.

TABLE II

Total Stem Length

(Cm. per plant.)

	Experiment 1. 12-hr. alternation.		Experiment 2. 1-min. alternation.		Experiment 3. Continuous light.	
Days.	A.	B.	A.	B.	A.	B.
2	9·95	—	8·10	—	5·90	—
4	12·61	—	9·87	—	7·46	—
6	11·83	11·35	8·88	—	8·20	8·79
8	12·49	13·08	9·84	—	—	10·14
10	15·25	15·07	11·55	—	—	11·70
12	17·14	17·37	13·31	—	13·25	13·49
14	20·36	20·01	15·74	—	15·76	15·56
16	22·61	23·06	17·01	—	18·16	17·95
18	26·73	26·58	18·80	—	20·49	20·70
20	29·12	30·63	19·40	—	—	—
22	37·08	35·30	21·28	—	—	—

Column A shows the experimental values, column B those calculated from the equation of the curve of closest fit. Extra soil was added to the pots after the fourth day, so that from the sixth day measurements were taken from the new soil-level.

shows, after a short initial phase of increase, a fall in growth rate with time. Although no investigations were carried out to test the point, it would appear probable that the production of growth hormones, as affected by the intermittent light, is concerned in the effects noted.

The actual experimental values obtained together with those calculated from the equations of the curves of closest fit are set out in Table II.

(c) *Increase in fresh and dry weight.*

The two curves for each of the three experiments as shown in Figs. 5 and 6 approximate closely to an exponential form, the calculated exponents having the relative order of magnitude expected. The logarithmic forms of the calculated curves of closest fit are:

Increase in Fresh Weight

12-hr. alternation: expt. 1. $\log_{10} W = 0{\cdot}0516t+2{\cdot}783$.
1-min. alternation: expt. 2. $\log_{10} W = 0{\cdot}0318t+2{\cdot}827$.
Continuous light: expt. 3. $\log_{10} W = 0{\cdot}0647t+2{\cdot}669$.

Increase in Dry Weight

12-hr. alternation: expt. 1. $\log_{10} D = 0{\cdot}0636t+1{\cdot}376$.
1-min. alternation: expt. 2. $\log_{10} D = 0{\cdot}033t+1{\cdot}465$.
Continuous light: expt. 3. $\log_{10} D = 0{\cdot}0648t+1{\cdot}399$.

Tables III and IV give the actual values obtained together with those calculated from the above equations for comparison. The discrepancies are not great when the small number of plants per sample is considered.

TABLE III

Total Fresh Weight

(Mg. per plant.)

	Experiment 1. 12-hr. alternation.		Experiment 2. 1-min. alternation.		Experiment 3. Continuous light.	
Days.	A.	B.	A.	B.	A.	B.
2	753	769	706	778	549	629
6	1308	1236	1129	1042	1308	1168
10	1979	1991	1440	1396	2223	2072
14	3123	3199	1923	1872	3747	3759
18	4881	5140	2516	2506	6379	6821
22	8641	8260	3201	3365	—	—

Values in column B are calculated from the equation of the curve of closest fit.

It is noteworthy that the increase in fresh and dry weight under intermittent light shows a constant relative rate and does not fall off as was found in the case of leaf-surface growth.

(d) *Reducing and total sugars.*

All sugar estimations were carried out by the method of Hagedorn and Jensen (1923), modified slightly in conformity with the experience gained in

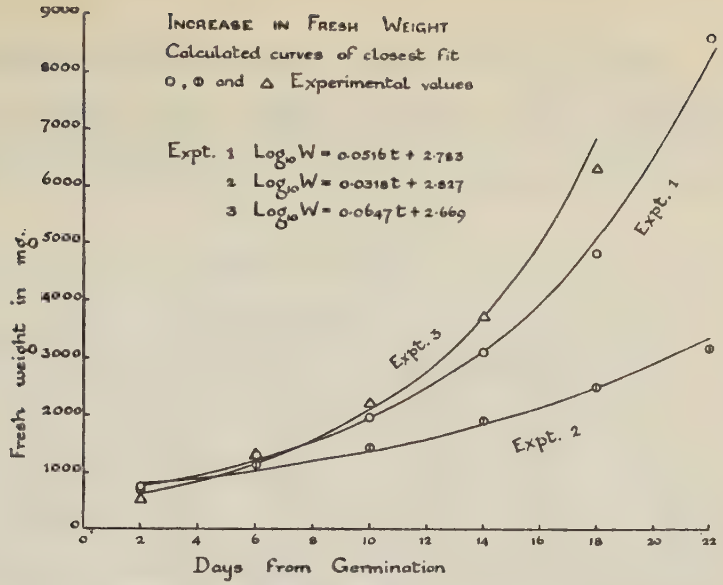

FIG. 5. Increase in fresh weight (mg.) of the whole plant; average values for random samples of four plants. Expt. 1, twelve-hour alternation; expt. 2, one-minute alternation; expt. 3, continuous light.

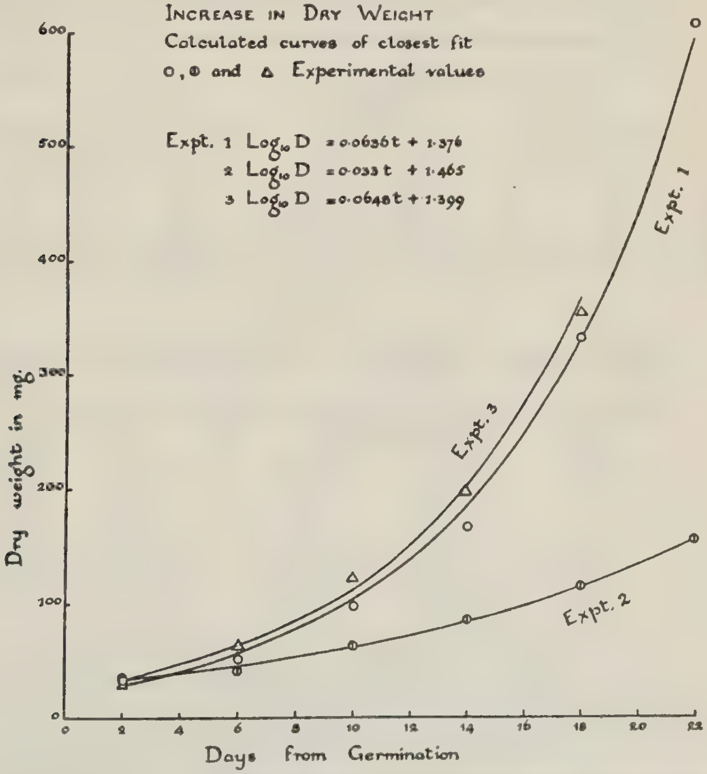

FIG. 6. Increase in total dry weight (mg.) of the whole plant; average values for random samples of four plants. Expt. 1, twelve-hour alternation; expt. 2, one-minute alternation; expt. 3, continuous light.

this Institute. Table V shows the changes in the reducing and total sugar content of the leaves and stems with time for experiments 1 and 2; no data, unfortunately, are available for experiment 3. There is little difference in sugar content of the stems in the two experiments. The leaves, on the contrary, show remarkable differences in total sugar content; the percentage total sugar remaining practically constant in experiment 1 with twelve-hour alternations of light and darkness, and falling off rapidly in experiment 2 under one-minute alternations. This can be readily accounted for by a study of Table VI which gives the percentage sugar content of the successive leaves

TABLE IV

Total Dry Weight

(Mg. per plant.)

	Experiment 1. 12-hr. alternation.		Experiment 2. 1-min. alternation.		Experiment 3. Continuous light.	
Days.	A.	B.	A.	B.	A.	B.
2	34·3	31·8	36·3	34·0	31·7	33·8
6	51·8	57·3	42·0	46·0	63·6	61·3
10	97·3	102·8	62·3	62·4	122·0	111·3
14	166·3	184·5	87·0	84·5	197·0	202·1
18	332·8	331·9	117·0	114·6	355·0	366·9
22	609·3	595·7	154·0	155·3	—	—

Values in column B are calculated from the equation of the curve of closest fit.

TABLE V

Sugar as Glucose. (Calculated as a Percentage of the Fresh Weight)

Experiment 1, 12-hour alternation; experiment 2, 1-minute alternation.

	Reducing sugar.				Total sugar.			
	Leaves.		Stems.		Leaves.		Stems.	
Days.	Expt. 1.	Expt. 2.	Expt. 1.	Expt. 2.	Expt. 1.	Expt. 2.	Expt. 1.	Expt. 2.
2	2·16	2·72	1·00	1·41	2·67	4·36	1·64	1·82
6	1·30	1·69	0·45	1·24	2·52	2·63	0·76	1·97
10	0·90	1·20	0·46	0·66	1·73	2·15	0·67	0·90
14	0·72	0·61	0·40	0·52	2·37	1·30	0·76	0·86
18	0·46	0·72	0·36	0·51	1·77	1·54	0·73	0·84
22	0·59	0·51	0·33	0·34	2·04	1·16	0·74	0·64

TABLE VI

Sugar as Glucose. (Calculated as a Percentage of the Fresh Weight)

Experiment 1, 12-hour alternation; experiment 2, 1-minute alternation.

Leaf number.	Reducing sugar.		Total sugar.	
	Expt. 1.	Expt. 2.	Expt. 1.	Expt. 2.
1	0·60	0·51	1·67	1·13
2	0·45	0·53	1·70	1·17
3	0·69	0·69	2·59	1·36
4	1·47	1·01	3·73	2·17
5	2·39	14·25	4·12	21·20
6	10·90	—	18·20	—

at the termination of the experiments, i.e. twenty-two days from germination, when it is evident that the older leaves in experiment 2 contain very much less total sugar than those in experiment 1.

4. DISCUSSION OF RESULTS

The effects of intermittent light of varying duration on the growth and development of the cucumber may be considered under two heads: firstly, the effect on the morphological structure and growth rate of the various organs, and secondly, the effect on the metabolic processes such as assimilation and respiration, though these effects are obviously interdependent.

Returning to the experimental data already presented, it is clear from the results of experiment 2 that very short alternate periods of light and darkness have a pronounced inhibitory effect on growth, both as regards height and increase in total leaf area. This reduction in the total leaf surface is brought about in two ways: (1) by a reduction in the maximal size of each leaf produced, as can be seen by inspection of Table VII, and (2) by delay in the unfolding of the successive leaves, only five leaves having appeared at the conclusion of experiment 2 as compared with seven in experiment 1. The reduced maximal size of the leaves under short-period intermittent light appears to be a consequence of smaller individual cell size rather than a reduction in the total number of cells produced.

TABLE VII

Maximal Area of Individual Leaves in Square Centimetres

	Experiment 1. 12-hour alternation.	Experiment 2. 1-minute alternation.
Cotyledons (2) . .	16·0	12·3
Leaf No. 1 . . .	24·0	12·3
Leaf No. 2 . . .	59·0	20·0

TABLE VIII

Net Assimilation calculated as Milligrams CO_2 per Square Decimetre of Leaf Surface per Hour Light

Period in days.	Experiment 1. 12-hour alternation.	Experiment 2. 1-minute alternation.	Experiment 3. Continuous light.
2–6	4·5	2·0	4·7
6–10	5·3	3·9	3·9
10–14	4·0	2·7	2·4
14–18	5·1	2·0	2·4
18–22	4·7	1·8	—

From the data of leaf area and dry weight the net assimilation rate was calculated over successive four-day periods by dividing the increase in weight of the whole plant by the average leaf area during each period. The results are presented in Table VIII.

Two main conclusions are apparent. (1) The assimilation rates per hour of light in both continuous light (experiment 3) and under one-minute alternations (experiment 2) are almost the same after the sixth day; they are much lower than those with twelve-hour alternations (experiment 1). Up to the sixth day, however, the assimilation rate in continuous light is the same as that with twelve-hour alternations. (2) The rate of assimilation remains nearly constant in the experiment with twelve-hour alternations, whereas with one-minute alternations there is a steady decline from the sixth day onwards. In continuous light a decline occurs also, but in the absence of a value for the final period it cannot be stated with certainty whether this decline in the assimilation rate continues or whether a steady rate of assimilation has been reached. It appears certain that the assimilation rate is much lower in continuous light than with twelve-hour alternations. Since, however, in the latter case the illumination period is half only that of continuous light, the total assimilation in twenty-four hours is almost the same in the two conditions. This is confirmed by the dry-weight increase (Table IV), the relative growth rates in the two cases being 0·064 and 0·065, respectively.

Comparing experiments 2 and 3, in which the assimilation rate is the same, and making allowance for the fact that the period of illumination in the former is only half that in the latter, it is clear that the total quantity assimilated in twenty-four hours will under one-minute alternations amount only to one-half that in continuous light. The relative rates of dry weight increase in these two experiments are 0·033 and 0·065, respectively, so that the dry weight accumulation is proportional to the net assimilation rate.

The data for sugar content are unfortunately not complete, as none are available for the continuous light experiment. The available data (Tables V and VI), however, show that the lower assimilation rate under one-minute alternations as compared with twelve hours is reflected by lower total sugar content in the leaves.

The data for leaf-surface growth show that the leaf growth rate is almost the same with twelve-hour alternations and with continuous light; it would appear, therefore, that carbohydrate supply is adequate to maintain leaf growth rate in these two cases at a level determined by nitrogen supply and temperature. As the temperature used was near the optimum found by Gregory (1928), it is probable that this growth rate was nearly maximal for the light intensity used. Under both conditions leaf growth has been shown to be exponential, the relative growth rate remaining constant. In experiment 2 with one-minute alternations the carbohydrate supply falls to half, and in this experiment leaf growth is not exponential and the relative leaf growth rate falls continuously. Under these conditions, therefore, it would appear that carbohydrate supply is not adequate.

The main effect, therefore, of the variation in the period of alternation is due to variations of net assimilation rate. As Gregory and Pearse (1937) have shown elsewhere stomatal closure occurs in short alternating periods of light and darkness, the maximal effect occurring in the neighbourhood of one minute. In order to test whether such an effect was operating in these experiments measurements of the stomatal aperture were made with a self-recording porometer, as described by Knight (1922). The results are given in Table IX.

TABLE IX

Relative Values of the Rate of Flow of Air through Equal Areas of Leaf Surface

	Experiment 1. 12-hour alternations.		Experiment 2. 1-minute alternations.		
	Light.	Dark.	Intermittent.	Light.	Dark.
	22·1	13·5	2·64	9·5	2·4
	48·2	8·8	3·65	—	—
	13·3	—	8·5	—	—
	32·4	—	9·0	—	—
Mean	29·0	11·2	5·95	9·5	2·4

It is clear that the stomatal aperture with one-minute alternations was very much reduced. It is reasonable, therefore, to assume that the differences in assimilation rate already referred to in experiments 1 and 2 were at least in part due to stomatal closure. No data are available for the experiment with continuous light.

In Table VIII it will be noted that during the period, 2–6 days, in which the cotyledons alone were expanding the net assimilation rate was the same in experiments 1 and 3 and much lower in experiment 2. The effect of one-minute alternations was therefore immediately apparent, whereas in the continuous light the assimilation rate was at first as high as with twelve-hour alternations. From this point onwards the assimilation was low, both in continuous light and with one-minute alternations. It would appear possible that the maintenance of a high assimilation rate requires a considerable period of darkness. The experiments of Garner and Allard (1931) would appear to support this, since with decreasing periods of darkness the amount of growth continuously fell off, which may have been due to a falling assimilation rate consequent on the diminishing period of darkness; in the absence of stomatal records for their work it is not possible to state this with certainty. On the basis of the data in the present experiments, it may be concluded with some certainty that the effects on vegetative growth noted by Garner and Allard were due to increasing carbohydrate deficiency occasioned by a falling net assimilation rate and partial closure of the stomata. The increase in growth obtained with more rapid alternations than one minute may have been due to increasing stomatal aperture as noted by Gregory and Pearse in *Pelargonium zonale*.

5. SUMMARY

Experiments are described in which cucumbers are grown under constant conditions of temperature and humidity with three different types of illumination: twelve-hour alternations of light and darkness, one-minute alternations of light and darkness, and continuous light. Measurements of leaf area and stem length were made throughout, and samples for fresh and dry weight and for sugar analysis were taken every four days.

Calculated curves of increase in total leaf surface and stem length are presented, together with comparative tables showing the measured and calculated values. Similar curves and tables for fresh and dry weight are given, together with tables showing the results of the sugar analyses.

The curves of increase in total leaf surface and stem length under very short periods (one-minute) of alternate light and darkness are found to differ markedly from the normal exponential curves obtained with long-period (twelve-hour) alternations or with continuous light. In the first case the relative growth rate of the leaf surface falls with time, whereas in the other two cases the relative rate remains constant. The curve of increase in stem length is sigmoid in the first case and exponential in the others.

The much lower growth rate found with short-period intermittent light is shown to be a consequence of (*a*) a reduction in the maximal size of the individual leaves produced, and (*b*) a delay in the unfolding of the successive leaves.

The curves of increase in fresh and dry weight are found to be exponential in form in all three experiments.

The main effect of variation in the period of alternation is found to be due to variation of net assimilation rate per unit area. Calculation of this rate over successive four-day periods leads to the following conclusions. (*a*) The assimilation rates in continuous light and under one-minute alternations are almost the same and much lower than with twelve-hour alternations. (*b*) The rate of assimilation remains nearly constant with twelve-hour alternations, whereas there is a decline in both the other cases. (*c*) The total amount assimilated in twenty-four hours per unit surface is almost the same under continuous light and with twelve-hour alternations. Only half this amount is assimilated with one-minute alternations. This result is confirmed by the dry-weight increases.

The carbohydrate supply appears to be adequate to maintain leaf growth rate at near the optimum both under continuous light and with twelve-hour alternations. It is not, however, adequate with the short-period alternations; this is confirmed by the much smaller amount of total sugar found in the leaves.

Observations of stomatal aperture taken with a recording porometer show part at least of the differences in assimilation rates to be due to closure of the stomata with one-minute alternations.

The necessity for a considerable period of darkness for the maintenance of high assimilation rates is put forward in explanation both of the effects recorded here and those observed by Garner and Allard.

In conclusion the author wishes to thank Dr. F. G. Gregory for suggesting the problem, and Dr. Sheng Han Shih for use of the data of the experiment with continuous light.

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The Interaction of Factors in the Growth of Lemna

X. The Interaction of Nitrogen and Light Intensity in Relation to Respiration

BY

H. L. WHITE

AND

W. G. TEMPLEMAN

(*From the Research Institute of Plant Physiology, Imperial College of Science and Technology, London*)

With nine Figures in the Text

INTRODUCTION

IN previous communications (Ashby, 1929; White, 1936 *b*) a technique is described whereby colonies of *Lemna minor* maintain steady vegetative increase for an apparently indefinite period under controlled conditions. The area of samples from these colonies is estimated by drawing an enlarged outline focused on a sheet of ground glass and tracing with a planimeter, as described by Ashby, Bolas, and Henderson (1928), and their dry weight by drying *in vacuo* under standard conditions and weighing on a microchemical balance, as described by Su and Ashby (1929). Allowance is made for the withdrawal of these samples when calculating the rate of increase of the colonies from daily counts of the number of 'fronds'.

In the present experiments respiration rates have been measured by a modification of a Warburg manometric method on samples corresponding with those used for area and weight determinations. By making the usual assumption that the rate of respiration is the same in the dark as in the light, the addition of the respiration values to determinations of net assimilation[1] provides estimates of the rate of real assimilation.

EXPERIMENTAL PROCEDURE

Lemna colonies, derived ultimately from the multiplication of a single plant, were grown under continuous illumination of 300, 600, 900, and 1,200 ft.-candles at a temperature of 25° C. The nutrient solution was made up with 'glass-distilled' water as follows: $CaH_4(PO_4)_2$ 0·101 gm.; $MgSO_4$ 0·255 gm.; $Mg(NO_3)_2 6H_2O$ 0·191 gm.; $Ca(NO_3)_2 4H_2O$ 0·290 gm.; K_2SO_4 0·334 gm.;

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[1] Calculated according to the formula given previously (White, 1936, p. 181) from increase in dry weight per unit area in unit time and converted into carbon dioxide on the assumption that the dry fond material is represented by the formula for starch or cellulose.

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Fe_2Cl_6 0·002 gm.; water 1 litre. It was impracticable to measure the respiration of more than one colony daily, and the respiration determinations cover a period of four days at the end of a period of nitrogen starvation (nitrogen content of nutrient solution 0·1 mg. per litre) and a similar period after the colonies had recovered from nitrogen starvation (nitrogen content of nutrient solution 50 mg. per litre) and were growing steadily at the same rate as before the period of starvation.

Each respiration sample of thirteen fronds of approximate area 0·8 sq. cm., together with a measured volume (0·2 c.c.) of the nutrient solution in which the colony had been growing, was transferred to a small glass cup standing on the floor of the respiration chamber above the level of a strong solution of potash. The respiration chamber and manometric system are represented diagrammatically in Fig. 1. The manometric system consists of the glass respiration chamber, about 3 c.c. capacity and varnished black on the outside, connected by fine capillary glass tubing and a three-way tap to the atmosphere and to coarse capillary glass tubing leading to a measuring burette with sliding lens outside the water-bath. After a preliminary period of thirty minutes to establish temperature equilibrium, the level of the liquid[1] in the manometric system is adjusted at atmospheric pressure to an arbitrary mark H and the tap G is closed. In accordance with an assumed respiratory quotient of unity the carbon dioxide emitted in respiration and absorbed by the strong potash solution is equivalent to the oxygen consumed, which is represented therefore by the change in pressure expressed by the change in level of the confining liquid in the burette after readjustment of the system of the respiratory chamber and capillary tubing to constant volume. As a blank was used in all experiments small variations in the temperature of the water-bath are immaterial. For the calculation of the oxygen of respiration from the change of pressure of the confining liquid the equation of Warburg (1930, p. 7, equation (3)) for a simple manometer has been used.

In all experiments respiration values for successive hours show a rise to a maximum and a subsequent fall. Curves of this type would arise if appreciable time elapsed before diffusion gradients of CO_2 between the plant and the absorbent were established. The shortness of the diffusion path and the exposed area of the absorbent, which covered the base of the respiration chamber, militate towards the establishment of rapid equilibria, but on the other hand the apparatus was not shaken and the samples were transferred to the respiration chamber with 0·2 c.c. of the nutrient solution in which the plant colonies had been growing. It may readily be calculated from the solubility of CO_2 and the respiration values obtained that an appreciable time elapses before the establishment of equilibrium between the CO_2 liberated in respiration and that dissolved by the nutrient solution. As the level of the CO_2 content of

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[1] Brodie's fluid was used, which has the following composition: 23 gm. sodium chloride; 5 gm. sodium cholate; 500 c.c. water; a few drops of alcholic thymol. 10,000 mm. of this fluid are equivalent to 760 mm. Hg.

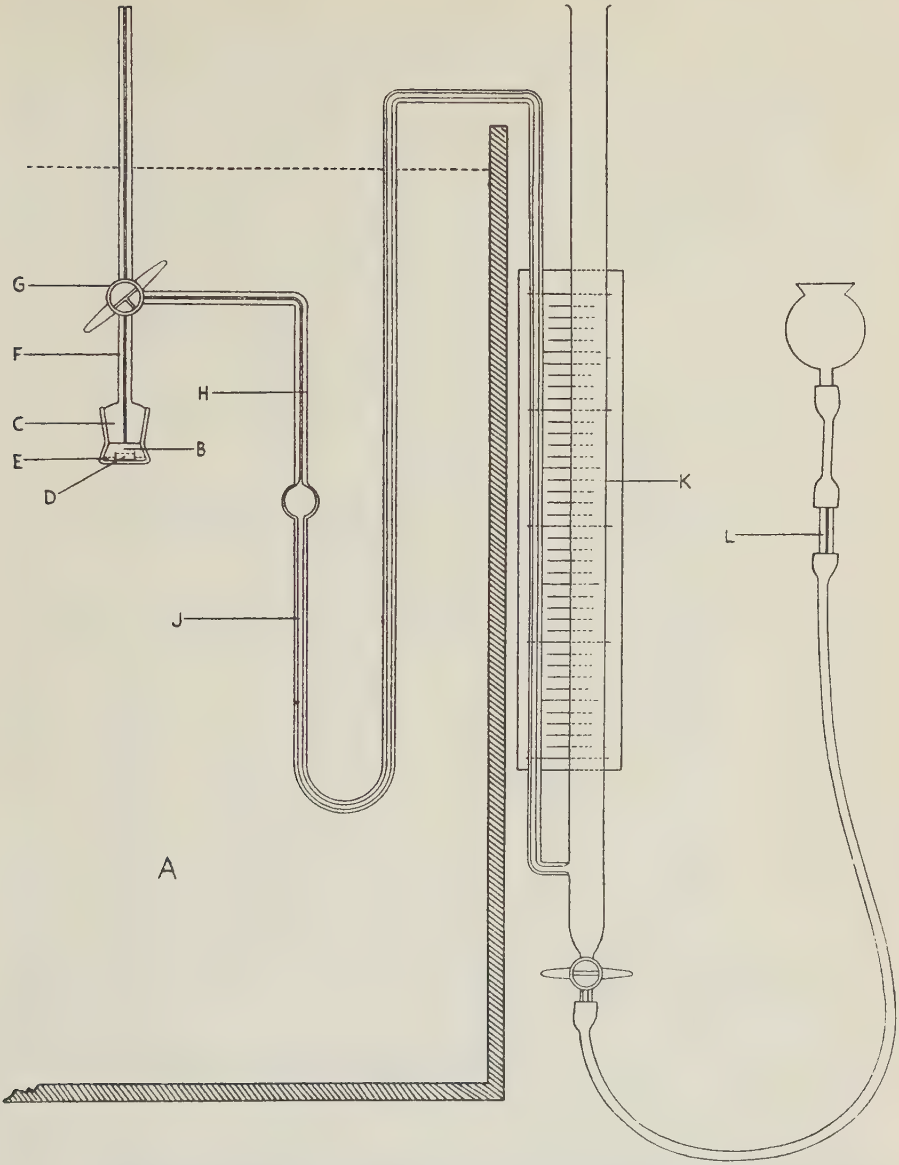

FIG. 1. Respiration chamber and manometric system. A. Water-bath containing toluene thermo-regulator, heating lamp, stirrer, and Beckmann thermometer. B. Respiration chamber. C. Ground-in stopper lubricated with vaseline. Respiration vessel is secured with rubber band twisted from base over three-way tap. D. Glass cup containing solution and Lemna. E. Potash (30%). F. Capillary tubing (area in cross-section 0·0489 sq. cm.). G. Three-way tap. H. Nick to mark limit of gas-system. J. Coarse capillary tubing. K. Burette with scale and sliding lens. L. Fine capillary as a resistance.

the solution rises more CO_2 escapes to reach the absorbent, leading to an apparent rise in respiration values over successive hours. The subsequent fall, similar to that found in many previous investigations, is attributed to the exhaustion of reserve carbohydrate. That the initial respiration values after transference to darkness are probably too low does not invalidate a comparison of the relative levels of respiration as between different treatments, for the maxima reached must be dependent on the differences in respiration levels determined by different treatments. This is apparent from the results obtained for the levels reached by the maxima of the curves are related to the times required to reach these maxima—a lower peak requiring a longer period.

EXPERIMENTAL RESULTS

The results have been calculated for each of nine hours from the beginning of the determinations and are given in Table I and Figs. 2 and 3. Figs. 2 and 3 show that respiration on an area basis rises with increase in light intensity. In Fig. 4 the mean respiration rates of hours 2–5 are used as a basis for comparison of the effect of nitrogen starvation. Fig. 4 shows that respiration is reduced by nitrogen starvation. Respiration rates have also been calculated on a dry weight basis. The results are given in Table II and Figs. 5 and 6,[1] which show that both with full nutrient supply and with nitrogen starvation the rate of respiration per gramme of dry weight increases as the light intensity is reduced from 900 to 300 ft.-candles. Although the values are somewhat irregular the fact that the curves at 300 and 900 ft.-candles do not intersect at any point on either diagram suffices to indicate a higher level of respiration for the colonies at low light intensity.

TABLE I

Respiration Rates on an Area Basis (mg. CO_2 per sq. dm. per hour) of Colonies under Continuous Illumination of from 300 to 1,200 Ft.-Candles during Nitrogen Starvation and with Full Nutrient Supply

(The figures in the first vertical column and horizontal row show respectively successive hours after transference to darkness and light intensity in ft.-candles.)

	Nitrogen starvation.				Full nutrient supply.			
	300.	600.	900.	1,200.	300.	600.	900.	1,200.
1	0·70	0·89	1·30	1·18	0·95	1·04	1·18	1·22
2	0·87	1·05	1·38	1·34	1·07	1·31	1·45	1·61
3	1·04	1·27	1·47	1·55	1·21	1·59	1·70	1·96
4	1·01	1·32	1·53	1·68	1·31	1·57	1·76	1·77
5	1·03	1·33	1·66	1·75	1·49	1·45	1·91	1·70
6	0·97	1·38	1·53	1·68	1·50	1·31	1·85	1·62
7	0·99	1·45	1·34	1·53	1·22	1·21	1·70	1·49
8	1·08	1·28	1·29	1·46	1·11	0·70	1·69	1·04
9	1·01	1·14	1·19	1·44	1·26	0·84	1·61	1·14

[1] The curves for 1,200 ft.-candles lie above those at 900 ft.-candles and are not plotted on the diagrams. Increase from 900 to 1,200 ft.-candles does not increase the multiplication rate of the colony in this experiment, showing that this range of light intensity is becoming supra-optimal.

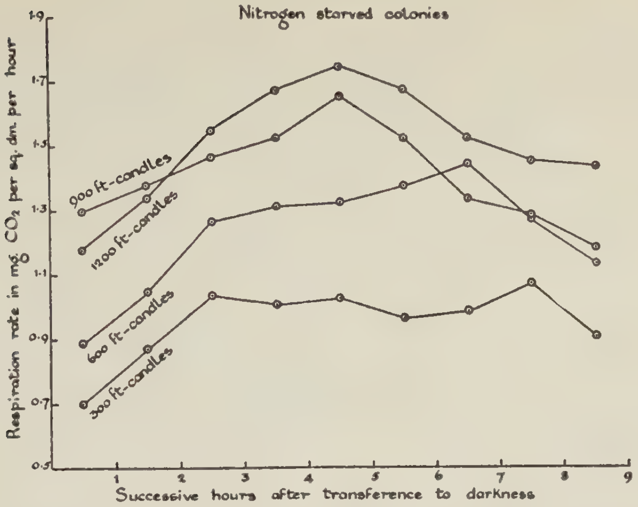

FIG. 2. Rates of respiration of nitrogen-starved colonies after transference to darkness from continuous illumination of four intensities.

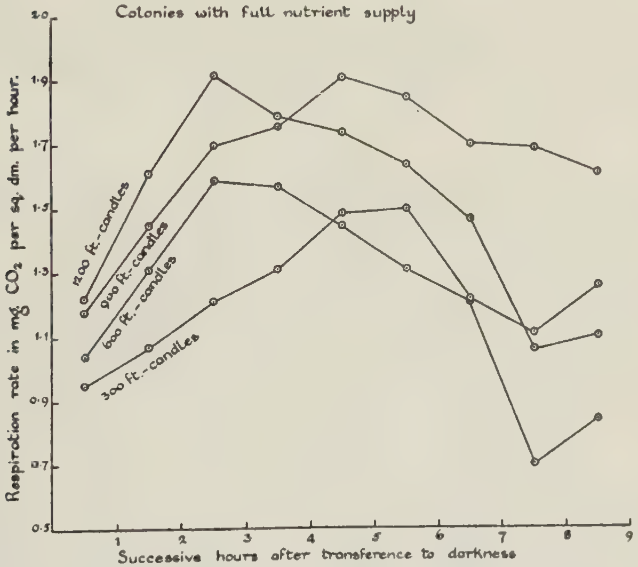

FIG. 3. Rates of respiration of colonies with full nutrient supply after transference to darkness from continuous illumination of four intensities.

Net assimilation rates, respiration rates, and calculated real assimilation rates[1] are shown in Table III and Figs. 7 and 8. The relationship between light intensity and both assimilation and respiration is of similar decrement type. By exterpolation of the curves a 'compensation point'[1] may be roughly estimated. Both with full nutrient supply and with nitrogen deficiency the value obtained is of the order of 45 ft.-candles, which would represent a minimal limit since it is suspected that the respiration rates are underestimated.

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[1] Net assimilation rates are calculated over the four-day periods corresponding to the respiration determinations. Mean rates for hours 2–5 are used as the basis of comparison of respiration.

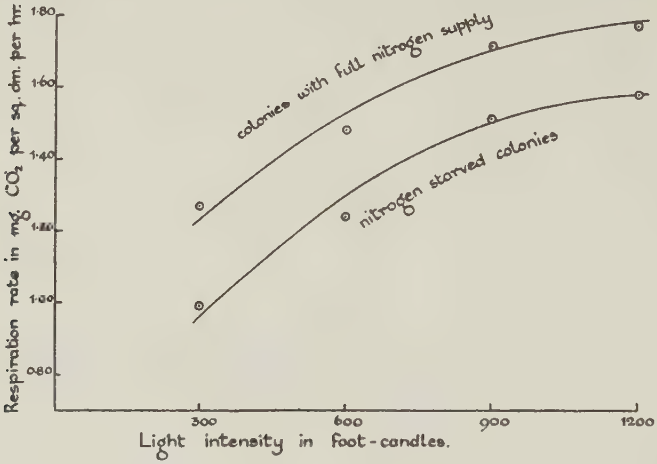

FIG. 4. Rates of respiration of (*a*) colonies with full nutrient supply, (*b*) nitrogen-starved colonies.

TABLE II

Respiration Rates on a Dry Weight Basis (mg. CO_2 per gm. dry weight per hour) of Colonies under Continuous Illumination of from 300 to 1,200 Ft.-Candles during Nitrogen Starvation and with Full Nutrient Supply

(The figures in the first vertical column and horizontal row show respectively successive hours after transference to darkness and light intensity in ft.-candles.)

	Nitrogen starvation.				Full nutrient supply.			
	300.	600.	900.	1,200.	300.	600.	900.	1,200.
1	3·46	2·94	3·41	3·65	5·39	4·86	4·32	4·82
2	4·31	3·46	3·62	4·15	6·08	6·12	5·31	6·37
3	5·15	4·19	3·85	4·81	6·87	7·43	6·23	7·59
4	5·00	4·36	3·96	5·21	7·45	7·34	6·45	7·08
5	5·10	4·37	4·34	5·42	8·47	6·77	7·00	6·88
6	4·81	4·56	4·00	5·21	8·52	6·12	6·78	6·48
7	4·90	4·79	3·50	4·74	6·94	5·66	6·23	5·81
8	5·34	4·22	3·37	4·52	6·31	3·27	6·19	4·07
9	5·00	3·77	2·99	4·46	7·14	3·93	5·90	4·35

From Table III it appears that respiration rates are not less than 30 per cent. of the real assimilation rates. The ratio between rates of respiration and those of assimilation is not appreciably altered by nitrogen starvation in this experiment. It may be noted that the net assimilation rates of the starved colonies are here *lower* than those of the colonies with full nutrient supply. They are falling steeply at the end of the period of nitrogen starvation. The net assimilation rates of more moderately starved colonies are characteristically *higher* than those of colonies with full nutrient supply (White, 1936 *a*), and determinations made in this experiment prior to the period of respiration measurement also showed this effect.

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[1] The point of balance between respiration and assimilation at which there is neither loss nor gain in weight.

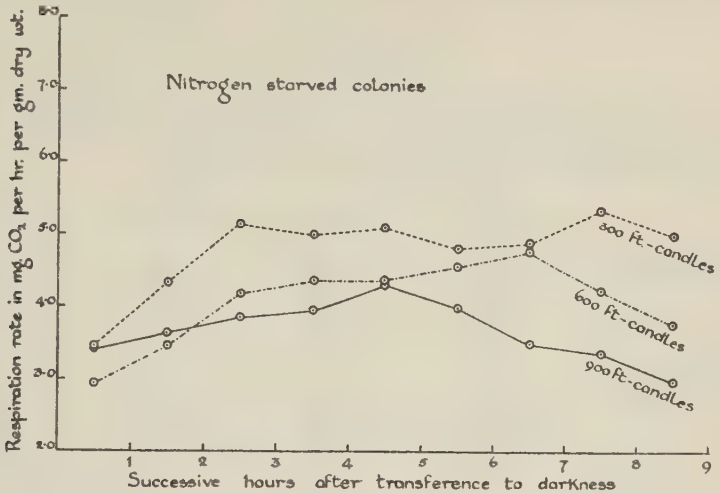

FIG. 5. Rates of respiration of nitrogen-starved colonies after transference to darkness from continuous illumination of four intensities.

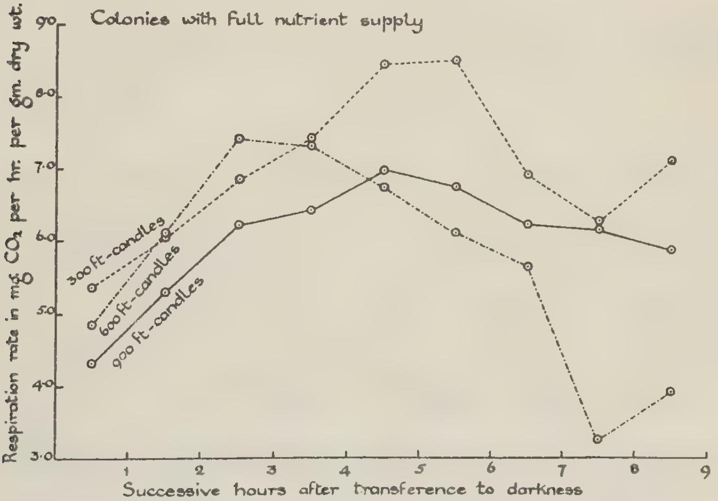

FIG. 6. Rates of respiration of colonies with full nutrient supply after transference to darkness from continuous illumination of four intensities.

The colony under 300 ft.-candles has the highest respiration rate relative to the real assimilation rate both with and without nitrogen starvation, though it cannot be determined whether this difference is significant. The results suggest that respiration is higher relative to real assimilation under low light intensities. In a previous communication (White, 1936 *b*) it has been shown that (1) there is an increase in amylolytic activity and a reduction in the starch content as the light intensity is reduced from 1,200 to 300 ft.-candles. It is probable that at low light intensities a higher percentage of the photosynthate formed is present as sugar and a lower percentage as starch and cellulose.

TABLE III

Respiration and Assimilation Rates (mg. CO_2 per sq. dm. per hour) of Colonies under Continuous Illumination of from 300 to 1,200 Ft.-candles during Nitrogen Starvation and with Full Nutrient Supply

	Net assimilation.	Respiration.	Real assimilation.	Respiration assimilation.
Nitrogen starvation.				
300 foot-candles	1·66	0·99	2·65	37·4%
600 ”	2·60	1·24	3·84	32·3%
900 ”	3·10	1·51	4·61	32·8%
1,200 ”	3·07	1·58	4·65	34·0%
Full nutrient supply.				
300 foot-candles	1·87	1·27	3·14	40·4%
600 ”	2·75	1·48	4·23	35·0%
900 ”	3·46	1·71	5·17	30·2%
1,200 ”	3·50	1·77	5·27	33·6%

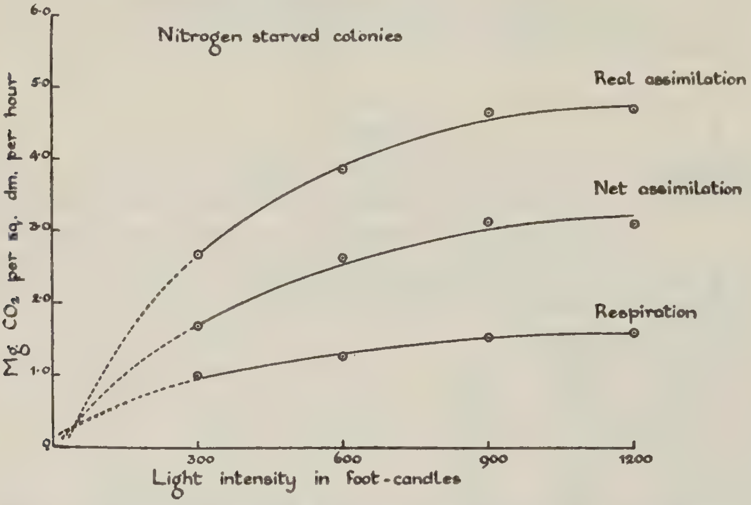

FIG. 7. Respiration rates, net assimilation rates, and calculated real assimilation rates of nitrogen-starved colonies under continuous illumination of four intensities.

(2) There is an increase in protein content (estimated from the staining of decolorized fronds with Millon's reagent) as the light intensity is reduced from 1,200 to 300 ft.-candles. Such changes would tend to make the rate of respiration at low light intensity higher relative to the rate of real assimilation. The increase in respiration per gramme of dry weight as the light intensity is reduced is clearly concordant with this view.

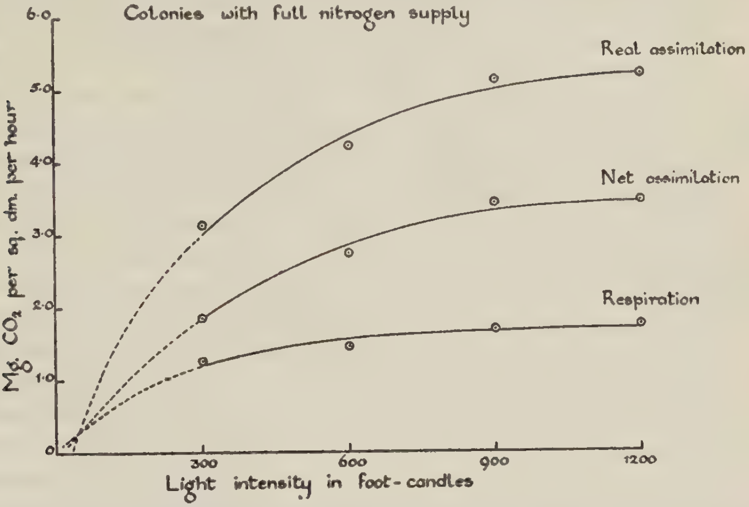

FIG. 8. Respiration rates, net assimilation rates and calculated real assimilation rates of colonies with full nutrient supply under continuous illumination of four intensities.

DISCUSSION

The effect of nitrogen supply and of light intensity on respiration is summarized as follows:

1. The rate of respiration calculated on either an area or dry weight basis is reduced by nitrogen starvation.

2. The rate of respiration on an area basis increases as the light intensity is *increased* from 300 to 1,200 ft.-candles. Since respiration is measured in darkness this is not a direct light effect, but presumably the result of an increase in carbohydrate content of the plants due to increased photosynthetic activity per unit area.

3. The rate of respiration on a dry weight basis increases as the light intensity is *decreased* from 900 to 300 ft.-candles. Dry weight per unit area rises steeply as the light intensity is increased (White, 1936 *b*, Table XIV), and if (as indicated on p. 198) a relatively high proportion of this increase is in non-respirable matter (cellulose) or reserve carbohydrate (starch), respiration on a dry weight basis would naturally fall with increase in light intensity in marked contrast to the rise shown on an area basis.

Respiration is thus related to two factors: (i) carbohydrate level, (ii) nitrogen content. Further evidence of the relationship of respiration to both these factors is provided by the respiration of a Lemna colony during a period of *recovery* from nitrogen starvation. During such a period there is a rise in nitrogen content,[1] resulting from transference of the starved colony to a solution with full nutrient supply, and a fall in carbohydrate level, as shown by a fall in dry weight per unit area from 4·01 to 2·39 mg. per sq. cm. (White, 1936 *a*, Table V). Daily tests with iodine also showed that surplus starch, accumulated during nitrogen starvation, disappears gradually during recovery. The rate of a process which is determined by the product of two factors, one falling and the other rising with time, would follow a curve that shows a maximum and subsequent decline. Respiration values, obtained by the manometric method for each of nine hours after transference to darkness and averaged to obtain a single daily value, are given in Table IV and Fig. 9. These values clearly show the rise and fall consistent with the relationship of respiration to both carbohydrate level and nitrogen content.

Carbohydrate accumulation has been shown to be symptomatic of nitrogen starvation (White, 1936 *a*). This is because nitrogen starvation affects markedly the rate of development of new fronds but does not reduce the assimilation rate. Consequently carbohydrates accumulate owing to non-utilization in growth. It follows that in nitrogen-starved fronds respiration would be affected by two opposing tendencies—that of reduced nitrogen content which would depress the respiration rate and that of increased carbohydrate concentration which would accelerate the respiration rate. Actual change in respiration rate would be dependent on the relative importance of these effects, and it is noteworthy that the depression of respiration during nitrogen starvation is only of the order of 25 per cent. (Fig. 4), whereas the rise in respiration rate, following transference of a starved colony to a solution with full nutrient supply, is of the order of 300 per cent. (Fig. 9).

4. Light intensity has a similar effect on assimilation and respiration not only with full nutrient supply but also with nitrogen starvation.

From the controlling influence of nitrogen supply on rate of increase in frond number and the independence of nitrogen supply and assimilation rate it is apparent that carbohydrate accumulation in nitrogen-starved fronds would be inversely related to nitrogen content. If nitrogen starvation were more severe under one light intensity than another a relatively greater depressant effect on respiration due to the low nitrogen content would tend to be balanced by an accelerating effect due to a correspondingly greater increase in carbohydrate content. The form of the curve of respiration under different light intensities would not be appreciably affected by nitrogen starvation, though the level might be lowered if the relative importance of the nitrogen factor rose with increasing intensity of nitrogen starvation. These expectations are realized in the experimental results, for the curve of respiration under different light intensities during nitrogen starvation is of the same form (Fig. 7) but at a lower level (Fig. 4) than that with full nutrient supply (Fig. 8), and further it has been found that (1) the fall in protein content (estimated from the staining of decolorized fronds with Millon's reagent) during nitrogen starvation is relatively greatest under the highest light intensity and less severe progressively with decreasing light intensity, (2) the increase in carbohydrate concentration, as estimated by relative rise in dry weight per unit area from that during the preceding period of full nutrient supply, was greatest under the highest light intensity and became less marked with decreasing light intensity.

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[1] Nightingale (1933) has shown that, on transference of nitrogen-starved tomato plants to a solution with full nutrient supply, nitrate was absorbed in a few hours but subsequent synthesis to protein occurred slowly. It is probable that recovery in the level of protein content in Lemna was also relatively slow, as the depth of (frond) colour, which has been correlated with protein content by Meyer (1918), Gassner and Goeze (1934), and others, and also in unpublished work in the present series of experiments, took several days to change from the pale yellow characteristic of nitrogen starvation to the deep green characteristic of ample nitrogen supply.

TABLE IV

Respiration Rates (mg CO_2 per sq. dm. per hour) of a Colony during Recovery from Nitrogen Starvation

Successive days beginning 30 Jan. 1933.

1	1·09
2	1·75
3	3·17
4	2·92
5	2·53
6	1·94
7	. .
8	1·66

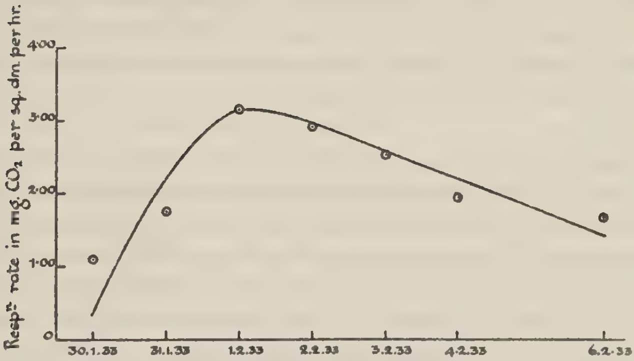

FIG. 9. Experimental respiration rates of a colony during the transition stage between nitrogen starvation and final recovery.

It is apparent that, as the intensity of nitrogen starvation increases respiration becomes controlled to a greater extent by the nitrogen content, so that even an increase in carbohydrate concentration equivalent to an increase in dry weight per unit area of 60 per cent. (White, 1936 *a*, Tables V and VI) does not raise the respiration rate to the normal level. On transference of a nitrogen-starved colony to a solution with full nutrient supply changes in metabolism occur which result in the loss, partly through an increase in growth-rate, but mainly through high respiration rate, of the whole of the surplus carbohydrate reserve accumulated during nitrogen starvation. These changes are not instantaneous, for there is a lag period of two days before the respiration rate begins to rise and the dry weight per unit area begins to fall, and moreover, there is some evidence (White, 1936 *a*, pp. 408, 412) to show that during this period the respiration rate falls lower and the dry weight per unit area rises higher than during the period of starvation. It is evident that an increase in *nitrate* supply does not immediately accelerate respiration and that the capacity for increased respiration rate is acquired slowly, as would be the case if it was dependent on the synthesis of a rising protein reserve. It is generally accepted that enzymes are of the nature of proteins or dependent for their functioning as part of a stable system on a proteinaceous substrate, and it has been shown (White 1936 *a*) that nitrogen-starved fronds are characterized by low amylolytic activity. These considerations favour the view that the low respiration of nitrogen-starved fronds is due to a low level of enzyme activity.

It is of interest to note that the general growth-activity of the colony, as estimated by the rate of formation of new fronds, does not exceed at any time during recovery the rate characteristic of a colony grown continuously with full nutrient supply. The multiplication rate is actually lower during the first few days of recovery than it is a week later, when respiration values have fallen to half their previous high values. Thus growth is at a low level at a time when respiration is at a very high level, and it appears that the two processes are not closely related. The very high respiration rate during recovery from nitrogen starvation, well above that characteristic of normal growth, shows that with ample nitrogen supply respiration tends to reflect the mass of respirable carbohydrate. This suggests that the close relationship between assimilation and respiration under different light intensities, demonstrated by the similarity in form of the curves of Figs. 7 and 8, arises through the influence of the concentration of photosynthate upon the rate of respiration.

Summary

The respiration of *Lemna minor* is estimated by a Warburg manometric method. A study is made of the effect of variation in light intensity on the respiration of (1) nitrogen-starved colonies, (2) colonies with full nutrient supply. Assimilation rates are calculated from increases in dry weight.

Respiration rate on either area or dry weight basis is reduced by nitrogen starvation.

Respiration rate on an area basis *rises* with increase in light intensity from 300 to 1,200 ft.-candles; respiration rate on a dry weight basis *falls* with

increase of light intensity. These contrasting effects are discussed. Rising respiration rate on an area basis is attributed to a rise in carbohydrate level associated with increased photosynthetic activity per unit area. Falling respiration rate on a dry weight basis is attributed to a relatively higher percentage of the photosynthate formed under high light intensities being transformed into non-respirable matter (cellulose) and reserve carbohydrate (starch).

Respiration is determined by both carbohydrate level and nitrogen content. Owing to the retarding effect of low nitrogen supply on rate of increase in frond number and the independence of nitrogen supply and assimilation rate, carbohydrates accumulate in nitrogen-starved fronds due to non-utilization in growth. The respiration of nitrogen-starved fronds is affected by two opposing tendencies—that of low nitrogen supply to depress respiration and that of high carbohydrate concentration to accelerate respiration. The curve of respiration of colonies with full nutrient supply under different light intensities is of the same form as that of real assimilation. The curve of respiration of nitrogen-starved colonies under different light intensities is also of similar form, but is at a lower level than that of colonies with full nutrient supply.

The processes of growth and respiration are not closely related; during recovery from nitrogen starvation respiration, influenced by a high carbohydrate reserve accumulated during starvation, rises to a level appreciably above that necessary for the maintenance of growth, which at this period is subnormal.

The mode of effect of nitrogen and light intensity on respiration is discussed. Depression of respiration by low nitrogen supply is attributed to a low level of enzyme activity associated with low protein content. The similar effect of light intensity on assimilation and respiration is attributed to the influence of the concentration of photosynthate on the rate of respiration.

In conclusion, the authors wish to express their indebtedness to Professor V. H. Blackman for the provision of facilities for carrying out the work and Dr. F. G. Gregory for suggesting an improved design of the apparatus used for estimating respiration and helpful criticism in the preparation of the paper.

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NOTE

With two Figures in the Text

AN INEXPENSIVE RECORDING MANOMETER.—The publication by Gregory and Pearse[1] of the technique for measuring stomatal movements by means of the recording porometer has marked a not inconsiderable advance in porometry. Not the least advantage of the method is that stomatal movements are recorded as such on a chart, and need no further working out, as is necessary with the more widely used Knight porometer. The method of recording used in the latter, though automatic, necessitates measurement of the recorded time-intervals, and the tedious plotting of these.

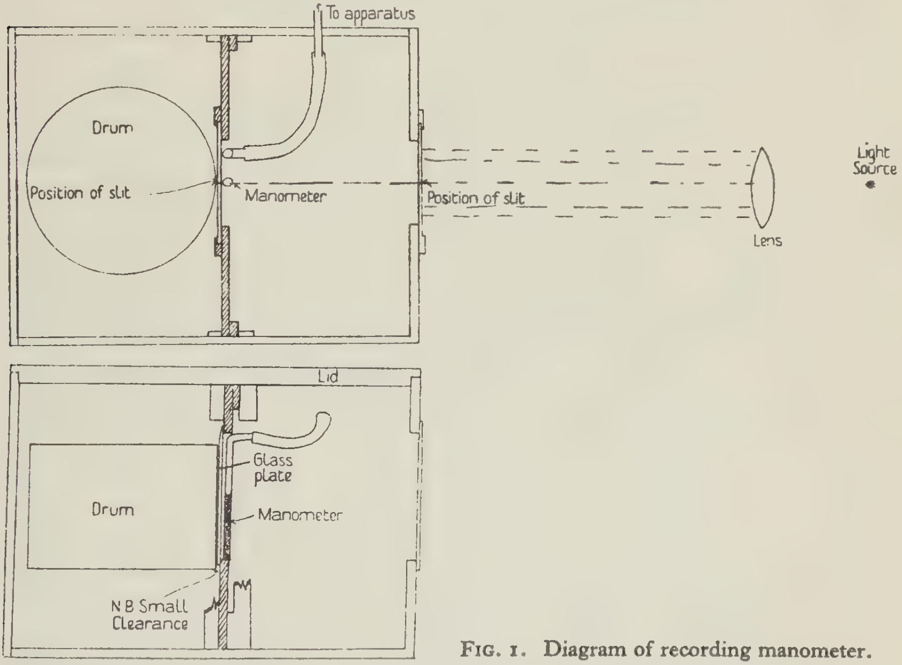

FIG. 1. Diagram of recording manometer.

Unfortunately, the recording manometer described in the original paper is expensive, as is the one evolved by Stiles and Leach.[2] There appears to be a need for a cheap instrument which will render the numerous advantages of the resistance porometer available to a wider circle of workers. The manometer to be described in this note has been in use in this laboratory for some months, and has given every satisfaction. It is easy to construct, while its cost is but a few shillings.

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[1] Gregory, F. G., and Pearse, H. L. The Resistance Porometer and its Application to the Study of Stomatal Movement. Proc. Roy. Soc. B, cxiv. 477, 1934.

[2] Stiles, W., and Leach, W. On the Use of the Katharometer for the Measurement of Respiration. Ann. Bot., xlv. 461, 1931.

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The principle is simple in the extreme. A narrow, parallel beam of light is passed through a manometer filled with a red solution. Immediately behind this is a revolving drum carrying bromide paper. The paper is unaffected by that portion of the beam which has passed through the red solution, and thus the manometric pressures are continuously recorded.

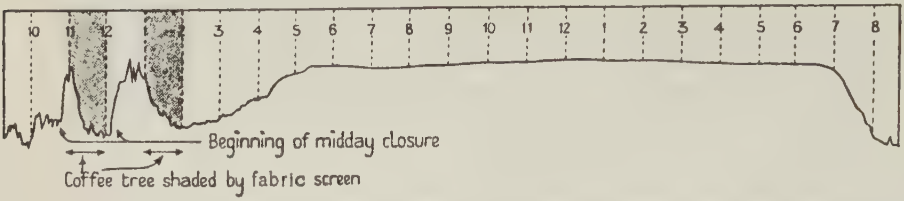

FIG. 2. Chart of variation of stomata of leaf of *Coffea arabica* obtained with the Resistance Porometer. On verandah; July 15, 1935; sunny day.

The apparatus consists of a light-tight plywood box, divided by a movable partition into two compartments. The rear compartment contains a recording drum. (The original was a twenty-four hour drum taken from an old thermograph. There is no reason, of course, why a drum of any other type should not be used.) The base of this is rigidly clamped in position. Immediately in front of the drum is a sliding partition, carrying, centrally, a blackened photographic plate, emulsion side towards the drum and almost in contact with it. A vertical scratch is made in the emulsion. The glass side of the plate carries the manometer, made of ordinary quill tubing. It is carefully alined with the scratch on the emulsion, and fixed firmly in position. The manometer communicates with the porometer by rubber tubing passing through a hole in the side of the box.

The front wall of the box carries a second photographic plate, with a slightly wider vertical scratch (0·5 mm.). The whole box rests on a base-board against three wooden pegs so that it can be removed and replaced in exactly the same position. The base-board carries a 6-volt motor head-light bulb, and a suitable lens. The apparatus is first alined so that the light from the lens passes through the two slits, throwing a narrow beam of light on the paper. The red solution is now introduced, and the apparatus is ready for use. The amount of extraneous light passing through the slits is insufficient to fog the paper, and the apparatus can be used in full sunlight.

A useful addition is to arrange an electrical contact so that the light is switched off for one minute by a relay at predetermined intervals. Thus the chart carries automatic time-markings. The works of a cheap alarm clock can readily be converted to this purpose.

The box can be removed from the remainder of the apparatus, carried to a dark-room, the chart changed and the old one developed. The total loss of time in the twenty-four hours is only a few minutes, so that the record is virtually continuous.

The chart reproduced shows, clearly enough, the type of record given by this instrument. It is accurate enough for all porometric purposes. For reproduction it is often convenient to ink in the curve and hour-markings with Indian ink; the silver then being removed by iodine followed by sodium hyposulphite.

EAST AFRICAN AGRICULTURAL RESEARCH STATION,
AMANI, TANGANYIKA TERRITORY.

F. J. NUTMAN.

Manganese Deficiency of Oats and its Relation to Soil Bacteria

BY

F. C. GERRETSEN

(*Director of the Microbiological Dept., Agric. Experiment Station, Groningen, Netherlands*)

With Plates V to VIII

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I. INTRODUCTION

Historical

NOTWITHSTANDING that as early as 1774 Scheele proved convincingly that plants absorb manganese from the soil, nearly 150 years elapsed before McHargue (1922), Gilbert and McLean (1928), Clark and Fly (1930, 1933), and others demonstrated by exact experiments that manganese was an indispensable constituent of green plants.

It is remarkable that though it was well known that the yields of different crops could be raised considerably by supplying manganese either to the soil or to the plants (Olaru in 1920), and that as a result the symptoms of certain diseases disappeared, such observations did not lead to the conclusion that in all these cases lack of manganese was the prime cause of the trouble.

In 1909 Sjollema and Hudig described a disease of oats, which they called the 'Veenkoloniale Haverziekte' (identical with grey speck disease), the occurrence of which they connected with prolonged alkaline manurial dressings; at the same time they suggested acid dressings and the application of manganese sulphate as the most effective means of curing the diseased plants. According to Krüger and Wimmer (1914) the disease resulted from 'injurious

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physiological reactions induced by the alkaline residues of certain chemical fertilizers. Söhngen (1914) was the first to suggest that this disease was caused by manganese deficiency in the plant. He suggested that the beneficial effect of acid fertilizers was not primarily due to the resulting lowering of the pH, but lay in the fact that at the lower pH values insoluble manganic oxides might be reduced to soluble manganous compounds by certain micro-organisms.

Another Dutch investigator, Aberson (1916), found nitrites in and around the roots of diseased plants, which led him to the conclusion that the real cause of the disease was to be sought in the presence of a poison produced by *Bacterium nitrosus*.

In 1919 Hudig and Meyer found that mixing a few grammes of the roots of diseased plants with the quartz sand in the culture vessel caused a severe outbreak of the disease, whereas mixing the sand with the leaves of healthy plants avoided it. They also pointed to the possibility that bacteria played an important role in bringing about the symptoms of the disease. In agreement with this assumption Aberson and Evermans (1927) found it possible to cure diseased soils by sterilizing them by heat. They also proved that the plants grown on these sterilized soils contained much more manganese, even ten times as much as those grown on the original soil.

By the fundamental research work done by Samuel and Piper (1928, 1929) it became possible to trace a path through the ever increasing and sometimes contradictory observations. In the first place they proved that the symptoms of the so-called grey speck disease were identical with those obtained by omitting manganese from different culture media. They also discovered that the disease was not limited to a pH of about 7·5, but that it occurred also in more acid culture media (pH 5·5) when sufficient care was taken to remove the manganese.

Sterilization of diseased soils by steam had a decided beneficial effect, but they showed that in this way considerable amounts of manganese were set free from the soil, even up to fourteen times the original amount. According to their observations sterilization with formalin was ineffectual, so they concluded that sterilization by heat worked only because of its action on the mineral constituents of the soil, and not by killing injurious micro-organisms (Samuel and Piper, 1928, p. 705). With regard to the cause of the disease they (1928, p. 799) remark: 'The disease would appear to result from unavailability of manganese, though the precise factors responsible for this non-availability are not yet known.' Attention is called to their observation that after water-logging diseased soils the disease disappears (confirmed by Godden and Grimmett, 1928), and that raising the pH of the diseased soils to 7·8 or 8 by adding alkali has the same effect. In accordance with the latter observation Maschhaupt (1934) as well as Popp (1934) noted a decided improvement by the application of unslaked lime to clay soils. Lundegårdh (1932) also demonstrated that the solubility of manganese in natural soils showed a minimum around the neutral point, coinciding with the greatest number of diseased plants in the field, whereas on the acid as well as on the alkaline side the solubility of the manganese increased and the grey speck disease diminished.

Lundegårdh's attempt to find a relationship between the ion-balance in the plant and the disease (already tried in 1914 by Kelley) did not lead to an explanation of the different phenomena connected with the disease. Contradictory to the observations of Samuel and Piper, that the manganese content of diseased plants is always less than 12–14 p.p.m. of dry matter, Lundegårdh found diseased plants with much more manganese (up to 420 p.p.m.) and healthy plants with less (down to 1 p.p.m.). His most important conclusion that manganese deficiency is not the only cause of the grey speck disease is fully supported by the investigations carried out by the author. That Popp *et al.* chose as a title for one of their recent publications 'Das Rätsel der Dörrfleckenkrankheit' points to the fact that observations have also been made elsewhere, which cannot be explained by manganese deficiency alone. That micro-organisms play an important role will be shown in the following pages.

The observation that the roots of plants deficient in manganese are often attacked by bacteria points to a relationship between plant resistance and manganese content. Moreover, no satisfactory explanation has hitherto been given for the occurrence of the typical spots on the leaves of diseased plants nor for the remarkable fact that grey speck disease often occurs on the border between healthy clay and healthy sandy soils. The author investigated some of these phenomena and has attempted to solve the problems, especially with regard to their mutual relationship.

Since the problem proved rather complicated it was unavoidable that the investigations remained in some respects incomplete or had to be limited to preliminary orientations.

The symptoms of manganese deficiency and of grey speck disease.

The nature of the symptoms depends largely on the quantity of manganese available to the plant and whether the roots are kept sterile or not.

In the beginning, when the manganese content is only slightly below the requirements of the plant, there is to be observed a slowing down of the growth rate of the leaves as well as of the roots. The external appearance of the plants is quite normal, leaf spots and chlorosis being absent. By supplying manganese the plants quickly recover. Also in the field a slight manganese deficiency does not necessarily give rise to the appearance of typical symptoms, even in those cases where a notable decrease in yield is to be observed. Rademacher (1935) working with a so-called resistant strain of oats 'Weissbulls Argus' noted a decrease in yield of 1,200 kg. grain (45 per cent.) and of 3,000 kg. straw (37·5 per cent.) on a diseased plot, compared with a near-by healthy plot, whereas leaf spots and other symptoms were scarcely to be observed.

When manganese deficiency is somewhat more pronounced the typical symptoms of grey speck disease appear; after six weeks the leaves become slightly chlorotic and show a number of infiltrated greyish-green spots, which dry up and soon become yellow-brown. At the same time the leaves often bend over sharply at these spots. It is important to note that though in more advanced stages chlorosis generally becomes severe and often a typical stripe chlorosis develops, a number of cases have been noted where many leaf spots were present, but hardly any chlorosis was observed.

In the case of pot cultures and water cultures the first leaves absorb the available manganese, and the manganese not being transported from one leaf to another, the younger leaves contain less and less. A serious chlorosis may develop, and soon necrosis of rows of cells situated between the green vascular bundles extends towards the top and the base of the leaf, finally leading to the death of the plant.

In the field, however, the plants often recover when they grow older. This might simply be a result of the more extensive root system, which is in contact with a larger soil area and consequently is able to absorb more manganese. It is also possible that the roots reach the subsoil, which is often less alkaline and contains more available manganese. Moreover, in the early stages of growth often a prolonged rainy period may set in in Holland by which the water content of the soil is raised to such a degree that microbiological reduction processes become possible; as a result sufficient manganese is brought into solution to bring the plants back to a healthy condition.

On well fertilized, mostly heavy soils, plants which recover show a pronounced anthocyanin-formation around the leaf spots already present. Hudig (l.c.) speaks in such a case of 'pseudo-grey speck disease', whereas Rademacher gives a more detailed description saying: 'Die Rotfleckigkeit ist eine besondere Form der Dörrfleckenkrankheit und tritt allein dort auf, wo die Pflanze sich in erfolgreicher Abwehrstellung befindet.'

The symptoms of extreme manganese deficiency are still more pronounced; the young plants droop and cease to grow after a few weeks. As soon as the manganese present in the seed is exhausted, the younger leaves show a pronounced stripe-chlorosis and are often aetiolated. Leaf spots appear, streaks of tissue collapse at the margins of the leaves, and a severe necrosis sets in which finally leads to the death of the plant. The investigations of the author generally do not relate to these extreme cases, which seldom occur in the field, at least in Holland. They relate to plants which had at their disposal manganese sufficient to prevent a premature death but small enough in amount to produce the typical symptoms.

Until now scarcely any attention has been paid to the root system of the diseased plants, notwithstanding the fact that a badly developed root system, with a number of decaying root-tips, is one of the most striking characteristics of more serious manganese deficiency.

When the roots are kept sterile plants deficient in manganese grow less vigorously and leaves and roots lag behind those of normal plants, but they remain healthy and generally the plants show no other symptoms of grey speck disease than a slight chlorosis, even when they contain *less* manganese than similar non-sterile, heavily diseased plants.

Low temperatures in the field as well as during pot experiments retard the growth of the harmful micro-organisms and consequently delay also the appearance of the typical symptoms.

II. STERILIZATION AND INFECTION EXPERIMENTS

Sterilizing with formalin a badly diseased sandy soil (Westerwolde, north of Groningen) resulted in a complete cure of the disease. Special care was taken to prevent reinfection during the evaporation of the formalin, which took place in an electrically heated small room, which had been disinfected with formalin. If this precaution is not taken reinfection can easily occur and the experiment fails.

TABLE I

The Effect of Formalin Sterilization. Sown June 12 (8 seeds per pot.)

Treatment.		Average height. cm.	Number of of grains per pot.	Dry wt. straw. (gm.)	Dry wt. grain. (gm.)	Remarks.
Original soil.	A.	52	27	3·4	1·0	All leaves show typical spots.
	B.	58	71	5·6	1·5	
Sterilized at 150° C.	A.	81	209	11·5	8·4	Plants healthy.
	B.	72	217	10·4	5·5	
Sterilized with formalin[1]	A.	89	346	18·5	12·2	Plants healthy, leaves dark green.
	B.	91	326	19·5	13·1	
Added 75 mg. $MnSO_4$ per pot.	A.	60	123	6·6	2·3	Plants small, stripe chlorosis, few leaf spots.
	B.	69	104	6·1	1·8	

A and B are duplicate pots.

The question arises whether this improvement has been caused by a liberation of manganese by the formalin treatment or by the sterilizing effect on injurious micro-organisms. The fact that the addition of 75 mg. $MnSO_4$ per pot gave a yield not one-third of that obtained with formalin proves at least that the success of the latter treatment did not depend only on the liberation of manganese. Of other possible explanations one may mention: (1) By the formalin treatment bacteria would be killed and the plant nutrients contained in their bodies set free. However, the liberation of these nutrients is the work of micro-organisms, and the medium being sterile this supposition is less plausible. (2) Micro-organisms of a parasitic nature may have something to do with the disease, these being killed by the formalin treatment. This hypothesis is most plausible and will be considered in detail in the following pages. (3) Micro-organisms of a non-parasitic nature, e.g. those precipitating manganese in the presence of organic substances (Söhngen, loc. cit., 1914), may be killed by the disinfectant, thus enabling the plant to absorb more manganese from the soil than from the non-sterile medium. The fact that the addition of such a large quantity of $MnSO_4$ as 75 mg. is not sufficient to cure the disease points to an immobilization of the manganese in the unsterilized soil during the experiment.

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[1] 5 kg. soil was mixed with 7·5 c.c. formol, dissolved in 150 c.c. water. After evaporation of the formalin the sterilized soil was put into sterilized pots and mixed with a fertilizer consisting of $NaNO_3$ 1 gm., $CaHPO_4$ 1 gm., KCl 0·5 gm., $MgSO_4$ 0·6 gm., $CaCO_3$ 4 gm., and 8 drops ferric chloride-citrate mixture containing 5% Fe. The constituents were sterilized separately in solution.

TABLE II

Manganese Content of Different Soils (mg. per kg. dry soil).

	Soluble in CO_2 free water.	Soluble in N/10 HNO_3.
a. Badly diseased, sandy soil, untreated . .	0·035	170
b. Sterilized with formalin.	0·032	162
c. Diseased, light clay soil (Groningen) untreated.	0·022	64·5
d. The same sterilized with formalin . . .	0·018	70·0
e. Healthy clay soil (North Groningen) untreated .	0·135	87·0
f. Sterilized with formalin.	0·146	88·0
g. Diseased garden soil, untreated, not fertilized .	0·024	—
h. The same after the addition of plant nutrients .	0·023	—
k. The same, not fertilized, sterilized with formalin	0·022	—

In order to determine whether the formalin treatment brings manganese into solution a number of soils were treated with formalin and after the disappearance of the latter the water-soluble manganese and the manganese extractable with a $\frac{1}{10}$ normal HNO_3 solution were determined. (Care has to be taken to neutralize the formalin and after the treatment to free the soil from all traces of formalin, to avoid the possibility of its acting as a reducing agent during the chemical determination.)

Formalin sterilization differs from sterilization by heat in not altering the available manganese content of these soils. (Steam sterilization of the diseased Mount Gambier soil, as shown by Samuel and Piper, raised the water-soluble Mn from 0·14 to 1·88 mg. and of the Penola soil from 0·016 to 0·060 mg.)

Steenbjerg (1934), investigating the action of reducing agents on grey speck diseased soils, also noticed a curative effect of the formalin treatment. A careful examination of his analyses shows that the effect of methol is due to its reducing action increasing the quantity of available manganese tenfold, whereas the formalin has a curative effect *without* altering the manganese content of the plants notably, as is shown in the next table.

Steenbjerg's results confirm those of the author with regard to the curative effect of formalin, though his interpretation is different and is not supported by his Mn determinations.

The most plausible explanation is that the curative effect of formalin is due to its sterilizing the soil and that micro-organisms play an important role in bringing about the symptoms of the grey speck disease.

TABLE III

After Steenbjerg

	Yield (gm. dry matter).	Mn (p.p.m. dry matter).	Exchangeable Mn (milli-mol. in 100 gm. dry soil.)
Untreated soil	32·2	11·4	0·06
494 mg. Mn added to 23 kg. soil	86·4	98·9	—
Soil treated with methol . .	104·7	134·5	—
Soil treated with formalin . .	81·7	13·5	0·04

Sterile sand cultures and infection experiments.

For the pot experiments quartz sand was raised to a red heat, treated with HCl, and washed with distilled water. After being sterilized dry at 150° C. the sand was mixed with the sterile culture solution containing in 4 kg. sand 1·3 gm. KNO_3; 0·3 gm. KH_2PO_4; 0·7 gm. Na_2HPO_4; 0·1 gm. $CaSO_4$; 0·1 gm. $MgSO_4$ 7 aq.; 5 drops ferricitrate (electrolytically prepared) and 0·2 c.c. of the Sommer-Sorokin mixture (1928) containing Cu, I and B (Mn omitted); the pH was 6·5. The sterilized pots were soaked in molten paraffin (enamelled pots are to be preferred).

The seed was sterilized with 1 per cent. germisan for two hours. After initial germination in sterile test-tubes with sand five seeds were planted in each pot and covered with a bacterial filter consisting of a layer of paraffined sand to break the capillary contact and a layer of paraffined cork-filings mixed with so-called 'katadyne-bolus' (an oligo-dynamic preparation consisting of diatomaceous earth with 10 per cent. silver) which prevents bacteria and molds from infecting the sand.[1]

Some of the pots were reinfected with increasing quantities of diseased soil. The results of the first experiment, which was run in duplicate, are summarized in Table IV.

Though as small a quantity as 0·5 per cent. is sufficient to call forth the symptoms, it is evident that they become worse with increasing infection. Practically there is no difference between the appearance of the plants on the original soil and those on the sand infected with 10 per cent. of the soil. To make certain that the outbreak of the disease was not due to some unknown constituent of the added soil, and that it was due to the living organisms, another experiment was made. The sterile sand was mixed with 5 per cent. diseased soil and compared with sand mixed with 5 per cent. of the same soil sterilized with formalin. At the same time sterile sand was infected with 0·5 gm. diseased root-tips of plants deficient in manganese.

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[1] For full particulars of the method see Gerretsen, 1935.

The yield of both sterile cultures Nos. I and IV (Table V) is much greater than that of the infected ones; mixing the sand with 5 per cent. sterilized diseased soil has rather a beneficial than a harmful effect. Attention is called to the fact that the healthy plants of No. I contain only 7·9 p.p.m. Mn whereas according to Samuel and Piper (1929, p. 519) oat plants require a minimum quantity of 14 parts per million for healthy growth. Most interesting is No. II; here we have a sterile culture which has been contaminated from the air during

TABLE IV

Oat Plants in Sand Culture

Treatment.	Condition of plants. After 4 weeks.	After 6 weeks.
1. Sterile sand . . .	Healthy, no symptoms.	Healthy, slightly chlorotic.
2. The same, infected with 0·01 % diseased soil.	,, ,,	,, ,,
3. Infected with 0·1 % diseased soil.	Healthy, one leaf with typical spots.	Plants stay somewhat behind, slightly chlorotic.
4. Infected with 0·5 % diseased soil.	First symptoms appear, 5 leaves diseased.	6 out of 10 plants show typical symptoms, however not seriously.
5. Infected with 2·5 % diseased soil.	Plants smaller, 10 leaves diseased.	9 out of 10 plants diseased 28 leaves show pronounced symptoms.
6. Infected with 5 % diseased soil.	Plants small, 7 leaves diseased.	All plants diseased, 33 leaves show pronounced symptoms.
7. Infected with 10 % diseased soil.	Plants small, 9 leaves diseased.	All plants badly diseased and in poor condition. 45 leaves show pronounced symptoms.

TABLE V

Treatment.	Dry wt. 10 plants (gm.).	Mn (p.p.m.).	Mn of culture medium (p.p.m.).	Condition of oat plants.
I. Sterile sand with-Mn.	12·8	7·9	8·3	Plants healthy, slightly chlorotic.
II. The same, spontaneously infected.	4·6	13·7	8·3	Plants small, badly diseased, heavy stripe chlorosis, many leaf spots.
III. The same, infected with 5 % diseased soil.	6·7	3·7	15·1	The same as No. II.
IV. Sterile sand with 5 % diseased soil, sterilized with formalin.	16·6	17·0	6·5	Plants healthy, leaves deep green, tillered better than No. I.
V. Sterile sand infected with 0·5 gm. diseased root-tips.	7·3	7·5	8·3	Plants small, with a few typical leaf spots.
VI. Diseased original soil.	9·3	5·5	16·5	Plants small, typically diseased.

the experiment, and from which three different species of bacteria could be isolated. Being exactly similar to No. I but for the infection, it gives a most convincing proof of the assumption that in a culture medium poor in manganese the outbreak of the typical grey speck disease may depend only on the presence of certain bacteria.

To obtain some idea of the manganese content of infected and sterile plants grown under exactly the same circumstances; the same experiment was repeated and each of the plants analysed singly (sown May 18, harvested June 22, after appearance of the first symptoms).

TABLE VI

I. Sterile sand. All plants healthy.		II. Sterile sand, infected with 5 % diseased soil. All plants diseased, showing typical grey speck symptoms.		III. Sterile sand, 20 mg. Mn added per 4 kg.; plants healthy.	
Dry wt. per plant. (mg.).	Mn (p.p.m.).	Dry wt. per plant. (mg.).	Mn (p.p.m.).	Dry wt. per plant. (mg.).	Mn (p.p.m.).
455	21·0	289	23·9	510	50·3
443	15·3	233	33·1	485	82·2
408	8·7	107	21·5	476	109·5
511	16·6	280	21·6	358	101·8
364	13·4	244	24·7	455	95·9
—	—	154	35·8	—	—
—	—	270	31·1	—	—
—	—	149	21·1	—	—
Mean 436	**15·0**	**216**	**26·6**	**457**	**87·7**

Manganese content per plant (mg.):
0·0065 0·0057 0·0401

The infection with 5 per cent. diseased soil reduced the dry weight of the plants to 50 per cent. of the sterile ones; the weight of the latter is nearly the same (95·5 per cent.) as that of the manganese plants, notwithstanding that the manganese content is only one-sixth. Attention is called to the exceptional fact that the manganese concentration of the healthy plants on sterile sand is smaller than that of the diseased infected plants. Generally it is to be expected that the sterile plants will contain more manganese, since the intact root system extends over a larger area of soil than the infected roots of the diseased plants and consequently is able to absorb more manganese than the latter. In this case, however, the quartz sand contained only a trace of manganese, and the limited quantity available has been distributed over a much greater quantity of plant material.

In the following case, where soil sterilized with formalin had been used, we see that the sterile plants contain more manganese than the non-sterile ones, notwithstanding that the manganese content of the soil remained unchanged.

Reinfection with 10 per cent. of the same unsterilized soil caused a decrease in yield of 41 per cent., the manganese content of the straw and grain dropped from 51·5 to 19·3 p.p.m. whereas the total manganese present in the plant was reduced by 78 per cent. The water-soluble Mn content of the soil before sterilization was 0·0185 mg. per kg. and after the formalin treatment 0·020 mg. per kg. As in the second case, 90 per cent. of the culture medium was exactly the same as in the first and only 10 per cent. of the *same* soil, though unsterilized, had been added, chemical action as a cause for this pronounced interference with the manganese intake of the plant must be excluded.

TABLE VII

Five grains per pot sown on May 5; harvested on July 24. Exp. in duplicate

	Yield, straw + grain, per pot. (dry wt.).	Mn content (p.p.m.)	Mn per plant. (mg.).	Condition of plants.
Diseased soil sterilized with formalin.	19·0 gm.	51·5	0·196	Healthy.
As above but reinfected with 10% original soil.	11·2 gm.	19·3	0·043	Typically diseased.

According to Söhngen (1914) and Beyerinck (1913) a number of micro-organisms are able to precipitate manganese as manganese-dioxide; it seems plausible that in the present case these micro-organisms play an important role, especially in the 'rhizosphere' of the more or less deteriorated roots where they find the organic material they want for their metabolism.

Further evidence will be given on page 225.

Sterile water cultures and infection experiments.

In order to observe the development of the roots and how they are affected by micro-organisms in the absence of manganese, a method of sterile water culturing was worked out (Gerretsen, 1935).

In the neck of a 1 litre Pyrex Erlenmeyer flask a glass tube (3·5 cm. diam. and 10 cm. long) is fastened by means of a rubber tube. The lower end of the glass tube, reaching just above the level of the culture solution, is closed with a piece of mosquito netting. The dehulled seed, after being sterilized with a 1 per cent. germisan solution for two hours, is put on the netting and covered with a layer (1½–2 cm.) of small glass beads. The upper end of the tube is closed with a cotton plug. As soon as the coleoptile reaches the cotton plug, the latter is removed and on the glass beads a bacteria-proof filter is brought, consisting of a layer of paraffined cork filings and a second layer of the same filings mixed with a silver preparation (katadyne-bolus, diatomaceous earth, covered with porous silver). The cork filings are pressed round the stem of the plant by a layer of katadyne-quartz. The plant grows undisturbed through the granular layers, whereas the intrusion of molds and bacteria is prevented by the oligodynamic action of the silver. (Care must be taken that direct sunlight does not shine on the region where the stem leaves the heat-insulating cork layer, in order to prevent injury of the plant by high temperature.) For the addition of culture solution the Erlenmeyer flask is connected to a sterile storage flask by means of glass and rubber tubes.)

The culture solution contained per litre: 0·5 gm. KNO_3; 0·25 gm. KH_2PO_4; 0·075 gm. $MgSO_4$; 0·10 gm. $CaSO_4$; 0·4 c.c. ferric chloride-citrate solution (electrolytically prepared), and 0·25 c.c. of the Sommer-Sorokin mixture containing traces of Cu, I, and B. By adding 0·55 c.c. 10 per cent. KOH the pH was made up to 6·4. The chemicals used had been recrystallized twice and the culture solutions contained only traces of manganese. In some cases these traces were successfully removed by a preliminary culture of oat plants; shaking the solution with 'norit' proved to be less effective.

TABLE VIII

Infection—Experiment. (June 20 to August 14)

Treatment.	Dry wt. (gm.). Straw.	Grain.	Total Mn in green parts. μgm.	Mn in plant (p.p.m.).	Condition of oat plants.
I. Sterile water culture.	1·3	0·2	15	14·0	Healthy, though small.
II. The same . .	3·0	0·65	22	9·1	,, ,,
III. 0·5 mg. Mn added as $MnSO_4$.	4·7	1·4	323	76·9	Healthy, robust plants.
IV. Infected with crude cultures from diseased root-tip.	1·7	0·04	14	10·4	Plants typically diseased, ears poor, several roots decaying.
V. Infected with diseased root-tip.	1·5	0·1	17	15·2	As above but symptoms more pronounced.

The manganese content of seed and solution was just sufficient to enable a single oat plant to remain healthy as long as the roots remained sterile. The leaves were smaller, sometimes more or less chlorotic, the roots were less developed, and tillering was markedly reduced. Infection with soil, with diseased root-tips, or even casual contamination from the air, gave rise to the typical grey speck disease symptoms.

On June 20 sterile water cultures of a number of oat plants were begun. The plants developed normally; on 20 July, when no symptoms of the disease were to be seen, two cultures were infected, one with a crushed diseased root-tip and another with a crude culture of bacteria isolated from such a root-tip. Eleven days later the plant infected with the root-tip became typically diseased, whereas it took a fortnight for the other infected plant to develop the first collapsed leaf spots. The sterile plants remained healthy until August 14, when the experiment closed; these plants however were smaller than those receiving 0·5 mg. Mn, the leaves were slightly chlorotic without stripes, the roots were healthy but less developed and tillering was reduced.

Special attention has to be paid to the root systems of the infected plants; it seems that the tips of the roots are very susceptible to bacterial attacks; they become glassy and slimy, mostly just above the water-level, and finally they totally decay. (See Pl. VI, Fig. 5.)

Bacterial cultures.

From the diseased root-tips a number of bacteria were isolated and some 30 strains were obtained in pure culture. A meat-broth medium proved to favour putrefying bacteria so that they overgrew the other micro-organisms. On a medium consisting of root extract (50 gm. air-dry oat roots extracted with 300 c.c. water, filtered and made up to 500 c.c. with 0·1 per cent. asparagin and 2 per cent. agar) the bacteria grew less vigorously and were easy to isolate.

Most frequently *Bact. fluorescens liq.* was found, as well as other putrefying bacteria; one receives the impression that there are a number of different bacteria which are able to attack the roots in the absence of manganese. Infections with pure cultures were not successful, but with crude cultures or mixtures of some of the bacteria isolated it was possible to induce grey-speck disease symptoms on manganese-deficient plants. This confirms the results of the preceding experiment: that in water cultures deficient in manganese oat plants remain healthy if the roots are kept sterile, but infection calls forth the typical symptoms. That the culture solution lacked manganese was proved by the fact that the addition of one mg. Mn more than doubled the weight of the plant.

The many diseased root-tips in the infected cultures illustrate that manganese has a decided influence on the resistance of the plant towards invading micro-organisms. Microscopical examination showed that often, especially in the beginning, the tips of the root hairs were occupied by bacterial colonies (see Plate VI, Fig. 8).

The number of these infection experiments is too small to allow any definite conclusions, especially with regard to the nature of the organisms. However, it seems probably that the grey speck disease is not a typical parasitic disease, but that we have to do with a reduced resistance of the roots caused by manganese deficiency, which enables certain saprophytic bacteria to act as parasites.

It is interesting to note that the oat plant is able to manage with a much smaller quantity of manganese under sterile conditions than in the presence of micro-organisms, an observation which has been made also by Clark and Fly with regard to Lemna (1931, 1933).

Aseptic cultures.

The question arises whether it would be possible to protect the root systems of manganese-deficient plants from attack by micro-organisms by the use of some mild disinfectant, and in this way to grow healthy plants on culture media poor in manganese. An attempt to do this by adding katadyne-quartz to the culture solution failed, because the silver was absorbed from the solution by the roots of the plants and its oligodynamic action nullified; moreover, the plants were poisoned.

Malachite green, methyl violet, and uspulun were toxic to the plant even in low concentration; germisan, however, gave hopeful results. In a preliminary experiment 0, 0·1, 0·05, 0·01, 0·005, 0·001, and 0·0005 per cent. germisan was added to the culture solutions. After six weeks the mean lengths of the plants were determined; they stood in the ratio of 100 : 12 : 29 : 57 : 104 : 121 : 95. Concentrations above 0·005 per cent. proved to be toxic, but at 0·001 per cent. some favourable effect seemed to be noticeable. The plants were better developed than the untreated ones and symptoms of the disease were absent. In the next experiment six oat plants were put in paraffined cylinders of 2·5-litre capacity. At the beginning of the experiment germisan was added in concentrations of, respectively, 0·0, 0·001, and 0·002 per cent., whereas 2 mg. Mn was added as $MnSO_4$ to one of the cylinders in order to test the Mn deficiency of the culture solution.

TABLE IX

Germisan Cultures

First experiment. 6 plants. 21 May–23 Aug.	Germisan (%).	Dry wt., 6 plants (gm.).			Remarks.
		Straw.	Grain.	Roots.	
I.	0·0	7·0	1·4	1·0	All plants severely diseased with typical symptoms.
II.	0·001	12·2	7·5	2·0	All plants healthy.
III.	0·002	10·5	7·9	3·0	As No. II.
IV. (2 mg. Mn).	0·0	9·1	1·8	2·2	Plants healthy, but grains formed later.
Second experiment. 6 July–23 Aug.			Total.		
V.	0·0	—	4·2	—	All plants diseased.
VI.	0·001	—	7·7	—	All plants healthy except for a few leaf spots.
VII. (1·5 mg. Mn).	0·0	—	17·7	—	Plants healthy.

Very soon the untreated plants lagged behind those treated with germisan and the Mn plants; typical grey-speck disease symptoms showed after a few weeks. Pl. VI, Fig. 6, shows the condition two months after the beginning of the experiment. The root systems of the untreated manganese-free plants were severely attacked and even coloured brown, whereas those of the germisan plants were for the greater part intact and pure white. Notwithstanding that the plants treated with 0·002 per cent. germisan looked better, the yield was inferior to that of the plants with 0·001 per cent. germisan.

The plants to which manganese was supplied were healthy; it is not clear why the yield in the first experiment is smaller than that in the second. In Table IX the results of two experiments have been summarized; the first was finished at the time the seed was formed, and the second was discontinued as soon as the differences had become sufficiently pronounced.

In all cases the addition of germisan has increased the yield considerably;

very remarkable is the influence on the weights of the roots, which in No. III are three times that of the untreated ones.

The germisan was tested for the presence of manganese, which proved to be absent. As the plants did not contain Hg the curative effect of the germisan is to be ascribed to its protective action on the root system preventing decay and allowing a vigorous development, even when only traces of manganese are present.

III. The Root System of Manganese-deficient Plants

Except for a few instances (McHargue, 1922, p. 1597) no attention has been paid to the appearance of the root system by the different investigators. The writer was struck by the fact that in sand cultures as well as in water cultures the root-tips of plants poor in manganese were very often decayed; they looked glassy, were often brown, and in more advanced stages they had completely disappeared. Pl. VI, Fig. 7, shows a few successive stages of disintegration. Microscopic examination shows that the attacked root-tips are surrounded by numerous micro-organisms, whereas in those cases where manganese had been supplied, root-tips and root-caps were intact, with only a few bacteria here and there.

It is interesting to note that the roots of sterile manganese-deficient plants are often covered with long root-hairs even in water cultures; after infection they disappear within a few weeks. In the first stages of Mn deficiency one can sometimes observe root-hairs with a typical bacterial colony at the tip, as is shown in Pl. VI, Fig. 8.

The decay of the root-tips prevents the further development of the roots; new roots are then developed which after some time follow the same course. Consequently the root systems of plants deficient in manganese remain small in the soil as well as in water cultures. This aggravates the condition of the plant because the smaller the root system, the lower the quantity of manganese it can absorb.

Though the writer does not exclude the possibility of necrosis of the leaf tissue independent of bacterial disorganization of the roots, especially in cases of extreme manganese deficiency, he has also observed numerous cases where the plant under normal conditions of infection becomes diseased, whereas it remains perfectly healthy with the same small amount of manganese when the roots are kept sterile.

With regard to the effect of the manganese content of the plant on its condition the following cases might be distinguished: (1) The quantity of manganese is below the minimum requirements of the plant, which dies soon after germination. (2) The quantity of manganese is just sufficient for the plant to grow under sterile conditions; the production of organic matter depends on the quantity of manganese present. Under normal conditions of infection the root-tips decay and the grey-speck disease symptoms appear. (3) The quantity of manganese is still below the optimum, the plant lags behind the normal plant, but does not show any typical symptoms of grey speck disease. (4) The quantity of manganese is optimal and the plant develops normally. (5) The quantity is above the maximum and the plant suffers more or less from chlorosis.

Bacterial counts in the root-tips of manganese-deficient plants.

When manganese deficiency reduces the resistance of the roots towards bacterial attack there must be a notable difference between the numbers of bacteria present in and on the roots of plants cultivated with and without manganese. To investigate this point the root-tips of a number of plants were cut off at a length of exactly 2 cm., ground in a mortar with the aid of diatomaceous earth and water, and washed with the rest of the 100 c.c. water into a flask; all ingredients being sterilized. This suspension was diluted 10- and 100-fold and of these dilutions 1 c.c. was mixed with a culture medium consisting of beef-extract (diluted 2 : 1), 0·5 per cent. glucose, 0·05 per cent. K_2HPO_4, and 2 per cent. agar. After three days at 25° C. the colonies were counted and compared with those originating from similar oat plants grown in the same culture solution to which had been added 1 : 500·000 Mn as $MnSO_4$.

TABLE X

Total Number of Bacteria Present in Root-tips of Oat Plants

	Mn deficient.	Mn added.
Water cultures 3 weeks old.		
1st root-tip	290000	72000
2nd ,,	332000	26000
3rd ,,	98000	50000
4th ,,	147000	30000
Mean	**217000**	**44500**
Water cultures 9 weeks old.		
1st root-tip	1640000	41000
2nd ,,	1300000	52000
3rd ,,	875000	200000
4th ,,	1690000	49000
5th ,,	1250000	30000
Mean	**1351000**	**316000**

It is evident that in both cases the numbers of bacteria present on the roots of the plants deficient in manganese are far in excess of those present on the roots of the manganese plants. This affords direct proof of the effect of the manganese content of the plant on its resistance to bacterial attack.

The presence of nitrites and ammonia in the roots.

The presence of such great numbers of bacteria in the root-tips combined with the easily decomposable material of the latter offers an opportunity for the reduction of nitrates to nitrites in the soil as well as in water cultures.

Aberson (1916) has repeatedly detected nitrites in and near the roots of diseased plants, and even considers the presence of the toxic nitrites as one of the principal causes of the disease. The writer has often found nitrites in non-sterile water or sand cultures deficient in manganese, whereas in those cases where manganese was added or the cultures were kept sterile no trace could be found.

In a number of cases the quantity of nitrites in the roots of diseased plants was determined and proved to be in the mean 1·9 mg. per kg. of roots (dry wt.), whereas in those cases where manganese was added the plants contained 0·8 mg. per kg. of roots. These quantities, however, are so small that they can give rise to no symptoms and are to be regarded as a secondary phenomenon.

TABLE XI

Ammonia Determination in Healthy and Diseased Oat Plants

		NH_3 (mg.) in 100 gm. dry plant material distilled with		
Condition of oat plants.		H_2O.	MgO.	Total.
1 *a*. Plants typically diseased.	I.	18·4	32·2	50·6
	II.	15·3	29·8	45·1
1 *b*. Normal, healthy plants from same soil type.	I.	0·0	11·6	11·6
	II.	0·0	13·2	13·2
2 *a*. Plants severely diseased.	I.	12·6	40·7	53·3
	II.	14·7	30·9	45·6
2 *b*. Healthy plants, a few roots slightly attacked.	I.	5·7	24·2	29·9
	II.	6·3	22·2	28·5
3 *a*. Plants diseased, alkaline soil.	I.	0·0	68·3	68·3
	II.	4·6	51·3	55·9
3 *b*. Healthy plants from same soil.		0·0	22·3	22·3
4. Healthy plants from acid soil.		0·0	4·6	4·6

The abundance of proteins in the root-tips may give rise to the production of ammonia by different micro-organisms. It was observed that when the roots of a recently diseased oat plant from a three-weeks-old water culture were put for one minute into a 0·1 per cent. solution of phenol red and then rinsed quickly with distilled water, the dye adhered to most of the root-tips, which were coloured deep red ($pH > 8·2$). Repeating the experiment with the roots of a healthy plant it was observed that the root-tips remained white. Though the Nessler reagent is not fully reliable in a case like this, it has often been observed that the root-tips of diseased plants turned brown when brought into this reagent, whereas those of healthy plants remained unchanged or became yellow. In order to clear up this point the ammonia was determined semi-microchemically; after rinsing the roots in distilled water and drying them quickly between filter-paper, the whole plant was cut into pieces; about 10 gm. of fresh material was mixed with 100 c.c. ammonia-free distilled water. At first 50 c.c. was distilled without any further addition to determine the free ammonia. The ammonia was absorbed in 5 c.c. $\frac{1}{70}$ normal HCl. Duplicate determinations were made on different portions of the material. After cooling, 0·5 gm. MgO was added and the solution was distilled *in vacuo* between 40° and 45° C. This is necessary to avoid destruction of the amino-acids.

The healthy plants contained an average of 17·1 mg. total NH_3 per 100 gm. dry wt., whereas the diseased plants contained 52·3. In order to check these results the NH_3 content of a few water cultures with and without manganese was determined in the culture solution as well as in the roots of the plants (culture sown Aug. 4, harvested Oct. 7).

TABLE XII

Ammonia Content of Culture Medium and of Roots. (Distilled at reduced pressure with alkaline methanol)

		Mg. NH_3 per 1,000 c.c.	Mg. NO_2 per 1,000 c.c.	Mg. NH_3 per 100 gm. roots (dry).
Mn-deficient culture, plants diseased.	I.	3·9	1·6	77·5
	II.	6·0	1·5	65·4
Mn-containing culture, plants healthy.	I.	0·20	0·03	24·1
	II.	0·32	0·04	18·0

The previous results are confirmed by this experiment; the quantity of ammonia in the diseased plants is so great that it might play some part in bringing about the symptoms of the disease.

Besides the production of ammonia by the bacteria, it is possible that ammonia is produced by the plant itself as a result of extreme carbohydrate hunger; this will be discussed later on in connexion with the influence of manganese on photosynthesis.

IV. THE LEAVES OF HEALTHY AND DISEASED OAT PLANTS

The presence of ammonia in the plant may have some influence on the reaction of the cell-sap. In order to investigate whether there existed some difference in the pH of diseased and healthy leaves, they were put into a 0·1 solution of phenol red. As a result a most unexpected difference between healthy and diseased leaves was detected. The leaves of healthy plants remained fresh and vigorous for 3–5 days although they had taken up the phenol red.[1] The leaves of diseased plants lose their turgor even in a few hours, become soft and shrivel within twenty-four hours. After forty-eight hours they are dried up (See Pl. VII, Figs. 9 and 10). Even healthy leaves of diseased plants behave in a similar way. The quick change in turgor points to an increase in the permeability of the protoplasm. The observation of Dr. W. Went (personal communication) that the permeability of different plant cells increases with reduced CO_2 assimilation, is extremely interesting, since reduced CO_2 assimilation is one of the most striking effects of manganese deficiency, as will be shown later.

The origin of the typical leaf spots.

A close examination of the leaves of diseased plants which have stood for some hours in phenol red often discloses the existence of small spots a few tenths of a mm. wide and 0·5–2 mm. long, which appear perfectly healthy, but in which the cell-sap is coloured deep red, indicating that the pH is above 8·4. Often these spots disappear within a few hours when the leaves stand in sunlight, whereas darkness seems to favour their appearance. Leaves of healthy plants do not show the phenomenon. In most cases the whole leaf so treated contains phenol red (yellow) in abundance, and the experiment proves that among numerous cells with acid contents there are a few with a pronounced alkaline reaction. Often the cells at the edge of the leaf behave in this manner, as is shown by the microphotograph (Pl. VII, Fig. 11).

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[1] The cell-sap being acid, the phenol red is yellow and invisible; by exposing the leaves a short time to NH_3 vapour they turn red in a very short time.

Phenol red accumulates around the leaf spots, perhaps as a result of increased evaporation from the affected cells. Generally the pH is below 6·8 (phenol red yellow); only at the beginning of the formation of the leaf spots, when the tissue has just collapsed, are the spots usually bordered by red edges, as is shown in the microphotograph (Pl. VII, Fig. 11). When the tissue is dead, the colour shifts back to yellow and the reaction is acid. It is tempting to link up the alkaline reaction in the cells with the bacteriological production of alkaline substances (ammonia?) in the roots. The following experiment supports this view. The roots of a recently attacked three-weeks-old oat plant from a Mn-deficient water culture were put into a 0·1 per cent. solution of phenol red, together with those of a normal plant of the same age. After forty hours the root-tips of the diseased plant proved to be brilliant red, and the dye could not be removed by rinsing with distilled water. The phenol red had penetrated into the plant, especially into the diseased leaves; the penetration could be made visible by exposing them to ammonia vapour. The roots of the healthy plant remained uncoloured and the plant did not contain even a trace of the dye.

In another experiment only one root-tip of a plant recently diseased showed signs of disorganization; after submergence of the whole root system for one minute in the phenol red solution and after rinsing with distilled water only the diseased root-tip proved to be red. The roots of the same plant were put for twenty-four hours in the phenol red solution, with the effect that the dyestuff accumulated round the few leaf spots, partly in the *red* configuration. The same experiment was repeated with a similar recently-diseased oat plant of which the decaying root-tip had been removed. The result was exactly the same, except for the colour of the phenol red accumulated round the leaf spots, which was without exception yellow instead of red. By cutting off the source of the alkali-production, the cell-sap had remained acid.

The question arises whether it would be possible to influence the appearance of the leaf spots by forcing the sap-stream along another track. This was achieved by making incisions in a number of *healthy* leaves of diseased plants. A decided influence of the incision on the appearance of the leaf spots was observed in a number of cases, as is shown in Pl. VII, Fig. 12. After a month the healthy leaf of a plant supplied with manganese showed no change; the leaves of the diseased plants, however, showed a clear zone above the incision. From the figure it can be seen that on the left-hand leaf the spots appear on one side beneath the incision, and on the opposite side above the incision. On the middle leaf the spots can be seen on both sides of the leaf below the incision, but on the right side only above the incision. It is evident that the sap-stream, before entering the clear zone immediately above the incision, yielded, through the anastomosing ramifications, the greater part of its alkaline contents to the numerous cells it passed through, causing a number of them to succumb, and in this way giving rise to the leaf spots. Attention has to be paid to the fact that the production of organic acids in the cells is limited by the reduced CO_2 assimilation in manganese-deficient plants, and consequently the neutralization of alkaline products will be prevented or reduced (Kultscher, 1931, 1932). In this way the conditions set up by the disorganization of the roots may be aggravated.

V. THE MICROBIOLOGICAL PRECIPITATION OF MANGANESE

The possibility of the microbiological precipitation of manganese has been demonstrated by Söhngen (1914) and by Beyerinck (1913). To investigate whether these processes played a role in the soil in relation to the occurrence of grey speck disease the following experiment was made: 30 gm. of a heavy clay soil containing $CaCO_3$ were rubbed down with 25 c.c. H_2O, mixed with 100 c.c. of quickly cooled water-agar (2 per cent.), and poured into a Petri dish (diam. 11 cm.). In the middle a hole, 2·5 cm. diam., was made and filled with a suspension of a sandy soil in agar containing 1 per cent. $MnSO_4$. After eight days at 25° C. a brown diffusion ring had been formed, 5·8 cm. in diameter. Mixing the soil-agar suspension with chloroform or keeping the Petri dish at 0° C. prevented the formation of the ring (Pl. VIII, Fig. 13). In the latter case a ring was formed one week after the Petri dish had been removed from the refrigerator and placed in an incubator at 25° C.

It is evident that this precipitation is caused by micro-organisms; in a number of cases brown spots of 0·5–1 mm. diameter were formed which proved to be colonies of bacteria in which MnO_2 had been precipitated. The quantity of insoluble manganese in such a ring was determined microchemically after removal of the water-soluble and exchangeable manganese, and proved to be eight times as high as in the original soil-agar suspension outside the ring.

From these rings and spots manganese-precipitating bacteria could easily be isolated on a culture medium containing 2 per cent. Ca-citrate, 0·2 per cent. $(NH_4)_2SO_4$, 0·5 per cent. $MnSO_4$, and a trace of $MgNH_4PO_4$, homogeneously suspended. Soluble phosphates are to be avoided because they precipitate the manganese beforehand and prevent the production of MnO_2.

In order to investigate the pH limits between which this precipitation takes place the same experiment was repeated with a clay soil-agar suspension practically free from $CaCO_3$, to which had been added increasing quantities of 0·1 N HCl or NaOH, giving a pH scale varying from 6·3 to 8·1. Ring formation was limited to the pH range between 6·5 and 7·8, being at a maximum about pH 7·0. Whether the precipitation of manganese outside these pH limits is prevented by purely chemical causes or is due to some specific action of the bacteria has still to be investigated. In some cases it was observed that the MnO_2 precipitated by the bacteria was dissolved as soon as the reaction of the culture medium became more acid by the accumulation of the metabolic products of the micro-organisms.

It is interesting to note that Lundegårdh (1932, p. 290) demonstrated that the manganese content of oat leaves showed a minimum on culture media with a pH between 6·5 and 7·5, whereas the greatest number of plants with grey speck disease was found on soils within the same pH limits, which coincided with a minimum of soluble manganese. Though the author does not exclude the possibility of pure chemical precipitation of manganese, it seems evident from these experiments that in the soil the manganese bacteria play an important role in converting soluble manganese into insoluble oxides. These processes are to be expected especially in the neighbourhood of the root, where easily decomposable organic matter is available. This is another reason why disease-producing soils lose their power on sterilization.[1]

VI. THE PHYSIOLOGICAL ROLE OF MANGANESE IN THE PLANT IN RELATION TO GREY SPECK DISEASE

The lowered resistance of the roots towards invading micro-organisms is one of the most striking features of manganese deficiency. Concerning the physiological role of manganese in the plant little is known; Miller (1931) remarks: 'The function performed by manganese in the metabolism of the plant rests almost entirely on theoretical grounds.'

A series of experiments on the physiological action of manganese were carried out by the author and are summarized here.[2] The quantity of carbon-dioxide assimilated in one hour at 25° C. under constant illumination by 100 sq. cm. of the leaf surface by intact leaves of oat plants deficient in manganese is less than half that assimilated by comparable leaves of normal plants. The redoxpotential of a suspension of crushed oat leaves in water shows a number of characteristic changes during illumination and shading. The influence of small quantities of CO_2 on the potential is far in excess of what might be expected from the change in pH, and it seems evident that the method enables one to study *in vitro* part of the process of photosynthesis. The rise in potential of the suspension of diseased leaves during illumination was generally much smaller than that of healthy leaves. In both cases it was observed that the addition of a few tenths of a mg. of manganese caused a considerable rise in potential, i.e. 250 mv. for the healthy leaves and 150 mv.

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[1] These experiments also give a plausible explanation for the occurrence of grey speck disease on the border between healthy (alkaline) clay soil and healthy (acid) sandy soil. Especially in this border we find a range of pH including those favourable for the microbiological precipitation of manganese. This results in the appearance of the grey speck disease on a limited strip between both soil types.

[2] A detailed report of the investigations will be published elsewhere.

for the diseased leaf. Most remarkable is the fact that the addition of manganese sulphate to the suspension before it had been illuminated did not markedly alter the potential, whereas with addition during illumination or immediately thereafter the potential rose considerably.

From these experiments the conclusion may be drawn that manganese plays an important role in the oxidation-reduction processes connected with photosynthesis, most probably in relation to the so-called 'Blackman reaction'. Lack of manganese results in a lowered CO_2 assimilation and consequently in a diminished production of leaves and grain. This also accounts for the low content of reducing sugars in roots and leaves as has been observed by Miller (1933) in the case of manganese-deficient wheat, corn, lettuce, and cabbage.

Since the roots receive less assimilates the root system remains small and microscopic examination shows that in a number of cases the cell-walls of the manganese-deficient roots are much thinner than those of normal plants. Often the root-tips are abnormally narrowed, indicating that cell division has been retarded; as a result the renewal of the shedded root-cap cells is delayed. Both facts favour the entrance of micro-organisms into the root-tips and give a simple explanation for the relation between manganese deficiency and the lowered resistance of the roots towards bacterial attacks.

VII. SUMMARY

1. Grey speck disease of oats is the result of a number of factors. It is not caused by manganese deficiency alone; the appearance of the typical symptoms depends, in addition, on the presence of certain bacteria which attack the roots of the plants.

2. The nature of the symptoms caused by manganese deficiency depends largely on the manganese content of the plant; when the amount of manganese is below the minimum the plant dies soon after germination by necrosis of the leaf tissue. With a minimum manganese content of from 5 to 35 p.p.m. the plant may remain healthy though small when the roots are kept sterile. Under normal conditions, which allow infection, the grey-speck disease symptoms appear.

3. Healthy plants can be grown on diseased soil which has been sterilized with formalin, a treatment which does not alter the manganese content of the soil.

4. Reinfection of sterilized soils with 5–10 per cent. of the original diseased soil causes the disease to reappear.

5. In sterile manganese-deficient culture media (quartz sand, water culture) healthy oat plants can be grown with a very low manganese content (5–10 p.p.m.). Infection of these culture media with a few diseased root-tips, or with mixed cultures of bacteria isolated from the latter, is followed by an outbreak of the typical grey speck disease.

6. By the addition of 0·001–0·002 per cent. of germisan to manganese-

deficient water cultures the development of the harmful root bacteria can be checked and the plants remain free from grey speck disease.

7. In a number of cases the ammonia content of manganese-deficient plants proved to be from two to three times that of normal plants; this is partly due to ammonia produced by micro-organisms attacking the root-tips, partly also by protoplasmic autolysis following extreme carbohydrate hunger caused by reduced photosynthesis.

8. These alkaline products, especially those transported by the sap-stream from the roots into the leaves, appear to be responsible for the typical leaf spots of grey speck disease.

9. The resistance of the root system to invading micro-organisms depends largely on the manganese content of the plant; the amount of manganese present determining whether certain bacteria act as saprophytes or as parasites.

10. Manganese plays an important role in the oxidation-reduction processes connected with photosynthesis. In manganese-deficient leaves the CO_2 assimilation may be reduced to half that of normal leaves. This accounts for the diminished yield, the reduced root system, and the lowered resistance of the roots to invading micro-organisms.

11. The precipitation of insoluble manganic oxides in the soil is caused by specific micro-organisms, between pH limits of 6·5–7·8. These limits coincide with those in the field within which grey speck disease is most frequent.

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EXPLANATION OF PLATES V TO VIII

Illustrating Dr. F. C. Gerretsen's paper on 'Manganese Deficiency of Oats and its Relation to Soil Bacteria'.

PLATE V

Fig. 1. Sterilization experiment. (*a*) Plants on diseased soil; (*b*) the same sterilized with formalin; (*c*) the same with the addition of $MnSO_4$.

Fig. 2. Infection experiment. Sterile Mn-free quartz sand, infected with increasing quantities (0–10 per cent.) of diseased soil.

Fig. 3. Infection experiment. (*a*) Sterile quartz sand, plants small but healthy; (*b*) the same infected with 5 per cent. diseased soil; (*c*) as (*a*) but after the addition of $MnSO_4$.

PLATE VI

Fig. 4. Sterile and infected water cultures of oats. (*a*) Mn-deficient culture solution, sterile; (*b*) the same after the addition of 0·5 mg. Mn as $MnSO_4$; (*c*) as (*a*) but infected with a crude culture of bacteria isolated from a diseased root-tip.

Fig. 5. The roots of two manganese-deficient plants. (*a*) The plant cultivated in a sterile

water culture; (*b*) a few weeks after infection with a mixed culture of two species of bacteria isolated from a diseased root-tip.

Fig. 6. The influence of a mild disinfectant on the development of oats in a non-sterile water culture. (*a*) The original culture solution without Mn; (*b*) the same solution without Mn but 0·001 per cent. germisan added; (*c*) the same solution without Mn but 0·002 per cent. germisan added; (*d*) the original culture solution without germisan but with 2 mg. Mn added.

Fig. 7. The root-tips of diseased oat plants in successive stages (right to left) of disorganization.

Fig. 8. Root-hairs with bacterial colonies. (From an infected Mn-deficient sand culture.)

PLATE VII

Figs. 9 and 10. A remarkable differentiation between the leaves of healthy and diseased oat plants. Fig. 9 (*a*) Healthy leaves of normal oat plants; (*b*) healthy leaves of Mn-deficient oat plants; (*c*) slightly diseased leaves of Mn-deficient oat plants after being put into a 0·1 per cent. phenol red solution. Fig. 10. (*a*), (*b*), and (*c*) show the same plants as those of Fig. 9 but twenty-four hours later.

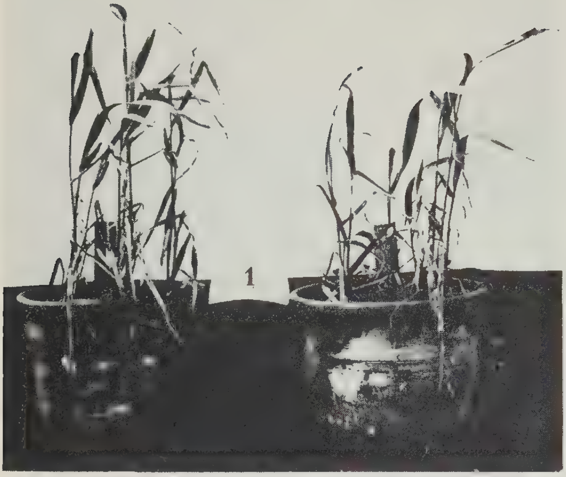
Fig. 11. Two leaf spots of oat plants at an early stage of disease. (*a*) At the dark area a new leaf spot is just being formed; here the phenol red, which penetrated into the cells, is brilliant red ($pH > 8{\cdot}4$), whereas in the surrounding cells it is yellow ($pH < 6{\cdot}8$): (*b*) a leaf spot a short time after its formation; at the edges of the spot phenol red has accumulated and the reaction of the cell-sap of rows of cells is alkaline.

Fig. 12. The effect of an incision on the appearance of the typical leaf spots. (*a*) Healthy leaf of normal plant, one month after the incision was made; (*b*) diseased leaf at the same time: below the incision leaf spots at both sides of the leaf, above the incision only at the right; (*c*) diseased leaf: the incision has forced the sap-stream to the right.

PLATE VIII

Fig. 13. The precipitation of manganese by micro-organisms. (*a*) Clay soil, suspended in agar; in the middle, sand in agar with 1 per cent. $MnSO_4$. After one week a brown diffusion ring of precipitated MnO_2 is formed. (*b*) To this dish chloroform had been added, which prevented microbiological precipitation.

Fig. 14. (*a*) Manganese dioxide containing bacterial colonies. (From a similar diffusion ring 10 ×.) (*b*) Manganese dioxide precipitated inside the mycelium of a fungus.



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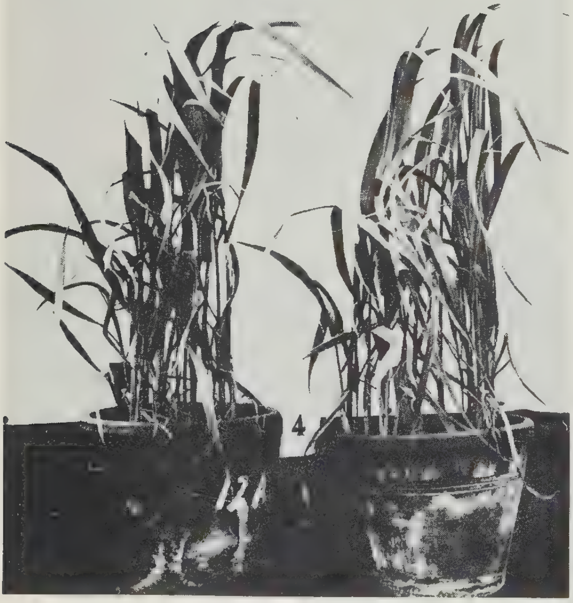

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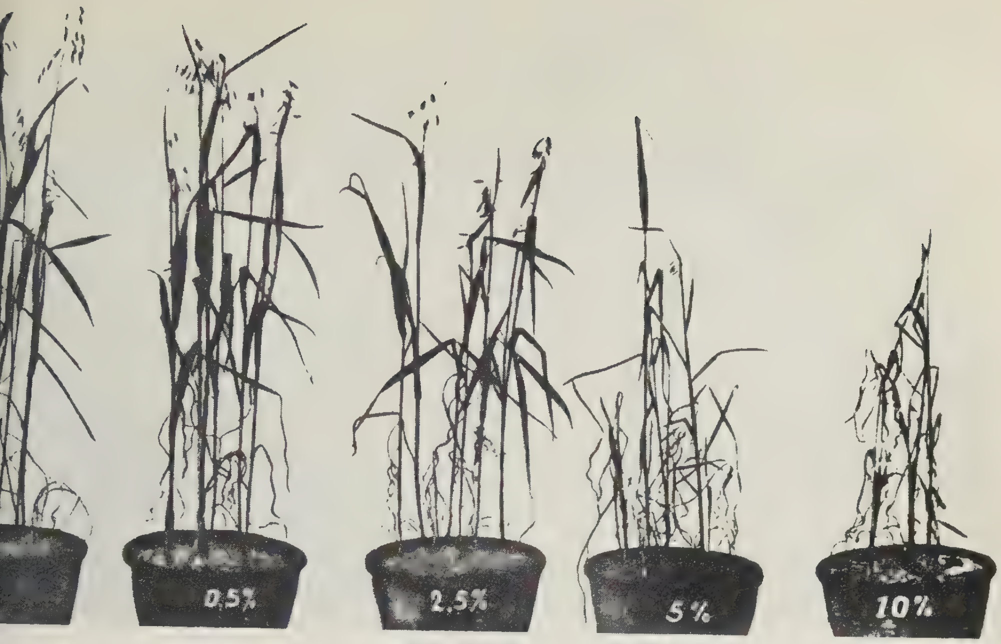
GERRETSEN – MANGANESE DEFICIENCY OF OATS IN RELATION TO SOIL BAC



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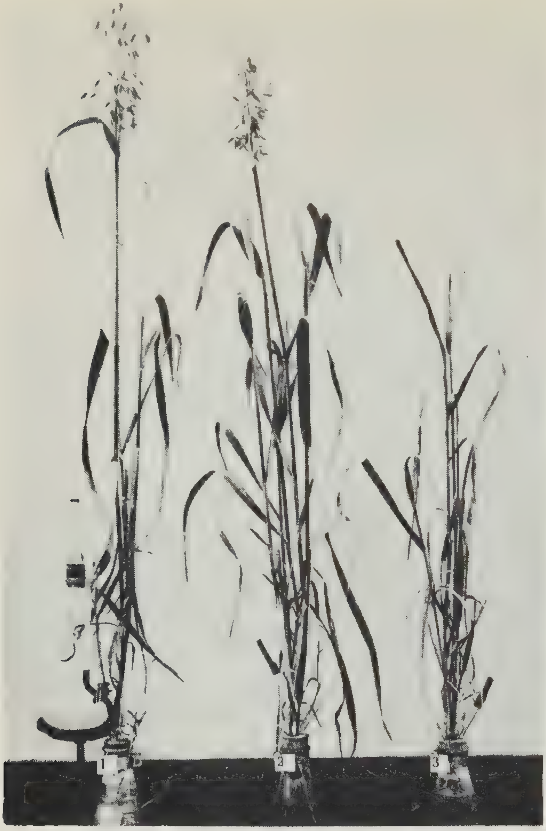

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GERRETSEN – MANGANESE DEFICIENCY OF OATS IN RELATION TO SOIL BACTERIA.

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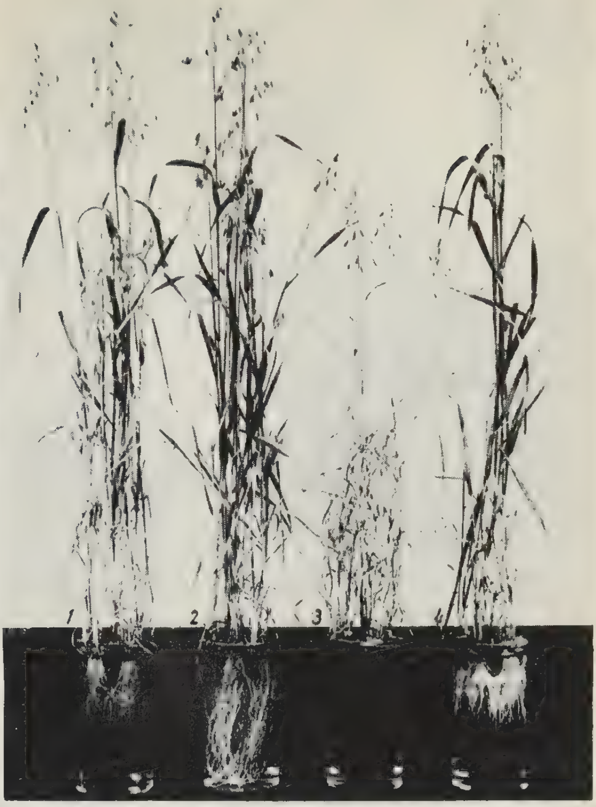

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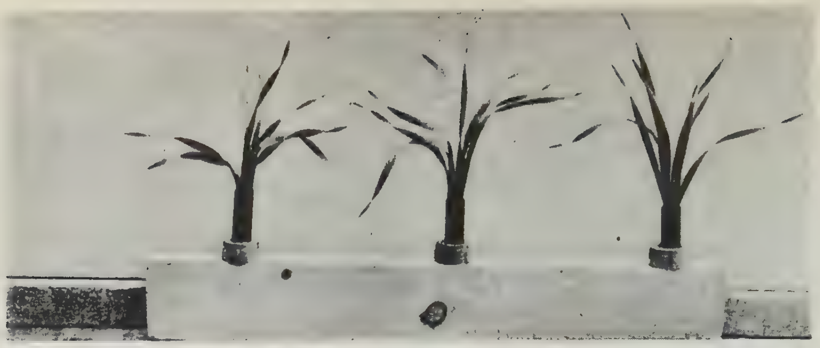

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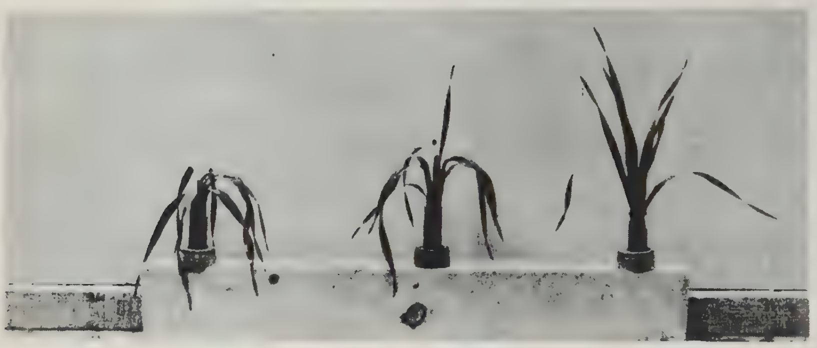

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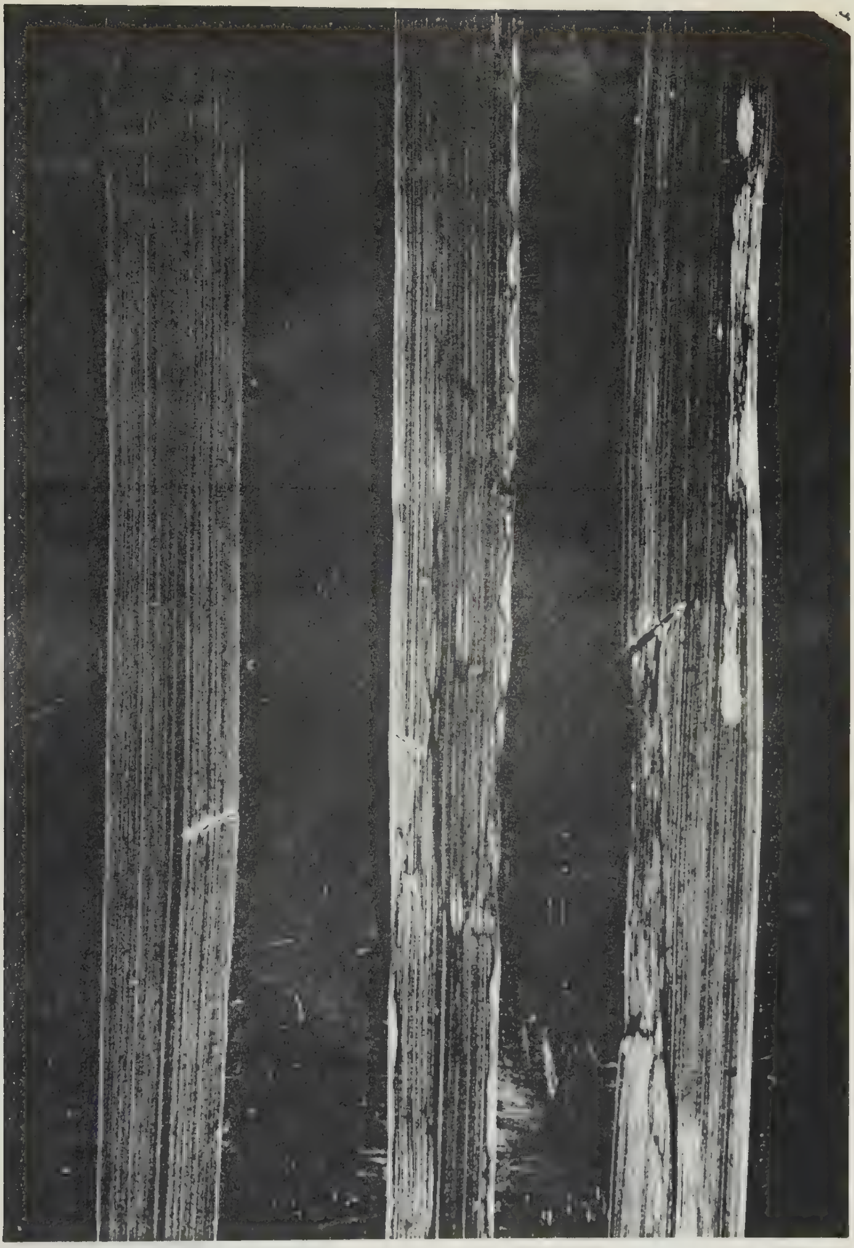

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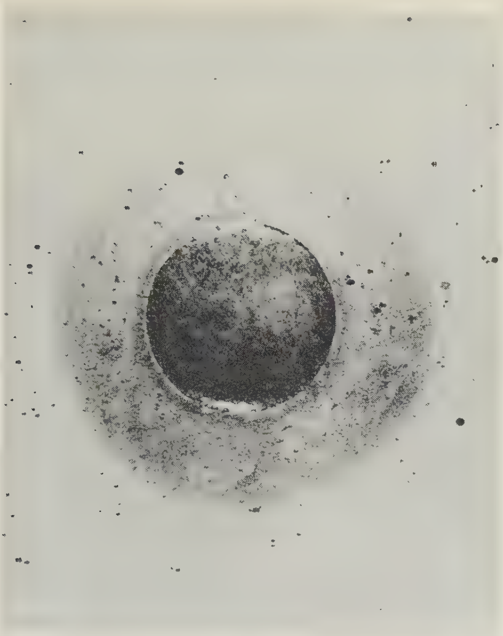

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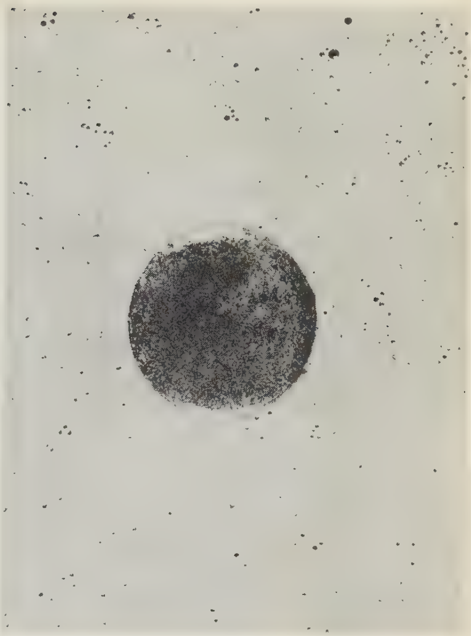

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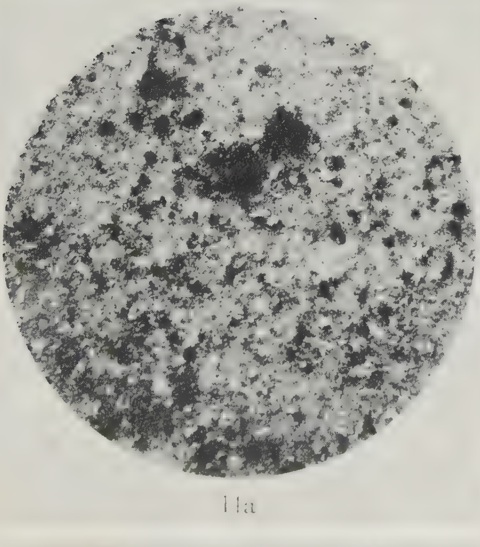

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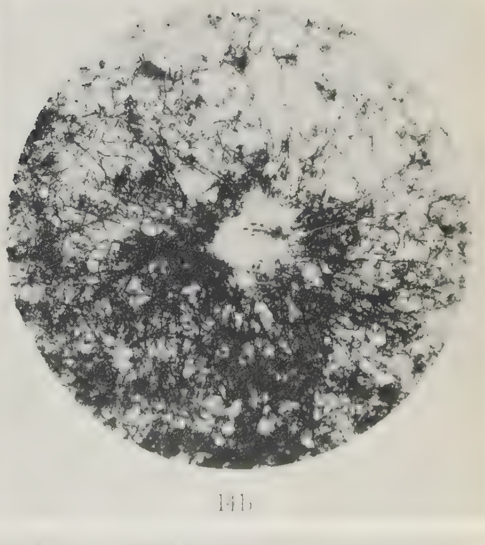

14b

GERRETSEN – MANGANESE DEFICIENCY OF OATS IN RELATION TO SOIL BACTERIA.

On the Effects of Light and of Oxygen on the Uptake of Sugar by the Foliage Leaf[1]

BY

E. PHILLIS

AND

T. G. MASON

(*Cotton Research Station, Trinidad, B.W.I.*)

With Plate IX

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I. INTRODUCTION

STARCH formation in leaves in contact with sugar solutions *in the dark* was first noted by Boehm (1883) and was confirmed by Meyer (1886). The procedure generally followed (cf. Parkin, 1889) was to float portions of leaves, previously destarched by darkening, on sugar solutions for a few days. The ability of several common sugars to produce starch was compared and sucrose was found to be pre-eminently the best. The lower limit of sucrose concentration at which starch formation occurred was 0·25 per cent. (Boehm, 1883), increasing concentrations causing increasing rates of starch production.

II. STARCH FORMATION

(a) *Light* (*Experiment 1*).

Attempts to form starch in cotton leaves by this procedure have given irregular but in the main negative results, in spite of the fact that such leaves readily form starch on insolation. It was discovered, however, that discs of

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[1] Paper No. 17 from the Physiological Department of the Cotton Research Station, Trinidad.

[Annals of Botany, N.S. Vol. I, No. 2, April 1937.]

destarched leaves immersed in sucrose solution readily form starch on exposure to diffuse daylight or to electric light of an intensity too low to form starch in the absence of the sugar solution. It seems clear that this effect of light was not due to photosynthesis, as starch was formed when the sugar solution and destarched leaf discs were placed in an atmosphere maintained free of carbon dioxide by potash solution. The sugar solution containing the discs was first placed for about eighteen hours in the dark to ensure the absorption of carbon dioxide and then exposed to diffuse daylight for twelve hours. The results of such treatment, using sucrose solutions of 2·5 and 10 per cent. and with controls kept the whole time in the dark, are shown in Plate IX. The discs were boiled for five minutes in water and then decolourized with alcohol. They were next dipped in boiling water and treated with a solution of iodine in potassium iodide. It will be seen that in the light all the discs formed starch, whereas in the dark only a small percentage did so. In the 10 per cent. 'dark group' only two discs formed appreciable amounts of starch. In the light, starch first appeared around the edge of the discs and along the veins and later spread across the whole disc. The formation of starch was of course much greater in the 10 per cent. than in the 2·5 per cent. solution. That carbon assimilation was in no way responsible for this effect of light is further emphasized by the way in which starch formation spread inwards from the periphery of the disc.

(b) *Oxygen* (*Experiment 2*).

Parkin (1899) noted that leaves floated on the surface of the solution formed starch, while those that sank to the bottom did not. He suggested that aeration was a factor in starch formation.

Our experiments have been carried out with *discs* floating on the sugar solution. In order to expedite the entry of sugar the discs were cut into annular rings as shown in Plate IX. The concentrations of sucrose (viz. 0·1 and 1·0 per cent.) used in this experiment were less than in the experiment just described. The sugar solutions containing the discs were put into closed vessels containing alkaline pyrogallol and alkali respectively. After standing for eighteen hours in the dark to ensure absorption of oxygen, the vessels of the Light group were exposed to diffuse daylight for a further period of twelve hours, after which the discs of all groups were examined for starch.

The results are shown in Plate IX. It will be seen that the Light-Air series shows most starch and the Dark-Nitrogen series the least, the other series being intermediate. It is noteworthy that a little starch was formed in the dark even in the absence of oxygen (note one small patch in the 1·0 per cent. nitrogen series), but more was formed in the light where, however, the absence of oxygen definitely retarded starch formation. Neither light nor oxygen would appear to be essential for starch formation, but they both independently greatly accelerate the process, so that the optimum conditions occur when both are present.

III. UPTAKE OF SUGAR

(a) *Light* (*Experiment 3*).

In experiments 1 and 2 it was shown that light promotes the formation of starch. In the latter experiment (Plate IX) it was shown that starch is formed from a concentration of sucrose as low as 0·1 per cent. The possibility arises that this formation of starch in light was due to the accumulation of sucrose against a gradient. Horn (1923) has shown that desiccation leads to the conversion of starch to sugar. There is also, therefore, the possibility that the observed formation of starch in light may have been due to an alteration in the starch-sugar equilibrium, either as a result of an increase in the water content of the colloids of the chloroplast or for reasons at present unknown. Our object in the present experiment has therefore been to ascertain whether light promotes the uptake of sugar by the leaf and whether this uptake takes place against a gradient.

TABLE I

Weights of Sugar, etc., per 100 Discs in Light and Darkness

	Gm.		Mg.		Gm. per 100 gm. water.	
Treatment.	Water.	Dry weight.	Reducing sugars.	Sucrose.	Reducing sugars.	Sucrose.
Initial *a*	2·49	0·550	4·14	0·18	0·166	0·007
b	2·49	0·563	3·36	0·18	0·135	0·007
Mean	*2·49*	*0·557*	*3·75*	*0·18*	*0·151*	*0·007*
Light *a*	3·52	0·478	5·00	3·45	0·142	0·098
b	3·69	0·492	4·81	3·26	0·130	0·088
Mean	*3·61*	*0·485*	*4·91*	*3·36*	*0·136*	*0·093*
Dark *a*	3·49	0·463	3·14	0·35	0·090	0·010
b	3·39	0·467	3·08	1·03	0·091	0·030
Mean	*3·44*	*0·465*	*3·11*	*0·69*	*0·091*	*0·020*

In this experiment the destarched leaf discs were immersed in 0·1 per cent. sucrose solution. The discs were cut into annular rings as in experiment 2. Each sample consisted of sixty discs, and at each collection duplicate samples were taken for analysis. There were three collections. An initial collection was taken before the discs were immersed in the sucrose solution and two were taken after immersion. The latter were first kept for eighteen hours in the dark in a carbon-dioxide-free atmosphere and one of them was then exposed for twelve hours to diffuse daylight, while the other remained in the dark as a control. The discs were dipped in water and dried between blotting-paper before sampling. The results are expressed on the sample basis and represent the weights of water, dry weight, and sugar per 100 discs. The sugars were extracted with boiling alcohol and determined by the Shaffer Hartman micro-method (Shaffer and Hartman, 1921).

The weights of material and the calculated concentrations of sucrose and reducing sugars are shown in Table I. It will be seen that both the Light and Dark collections show an increase in water and a loss in dry weight on the Initial collection. The Light group has, however, a greater dry weight than the Dark, and as it has also a greater weight of sucrose and of reducing sugars, it seems clear that light has promoted the uptake of sugar by the leaf.

From the data in the table it is not evident that there was any uptake of sugar by the Dark group, but as the Dark group in experiment 2 (cf. Plate IX, Dark-Air series) showed some starch formed in the 0·1 per cent. sucrose solution and none in water, it might be inferred that this was so. The losses in dry weight most probably occurred as a result of respiration and leaching during the initial period of eighteen hours' darkness.

TABLE II

Estimated Sugar Concentrations in Cells of Leaf

Gm. per 100 gm. water.

Treatment.	Reducing sugars.	Sucrose.
Initial . .	0·151	0·007
Light . .	0·197	0·090
Dark . .	0·125	−0·010

We have now to consider whether sucrose entered the Light group against or with the sucrose gradient. The concentration in the external solution was 0·1 per cent. and in the discs 0·093 per cent. The increase in the weight of water that took place between the initial and final collections may have been partly or altogether in the intercellular spaces. If it was altogether in the intercellular spaces the concentration in the tissues of the leaf can be corrected for the 0·1 per cent. sucrose solution in the intercellular spaces. These corrected concentrations are shown in the Table II. It will be seen that this correction makes but little difference to the sucrose concentration, but that the concentration of reducing sugars is appreciably increased. It seems probable that these concentrations should be raised by about 10 per cent. for bound water (Mason and Phillis, 1936*b*). On the other hand, as the concentration of sucrose is much less in the mesophyll than in the vein, the actual concentration of sucrose in the mesophyll would be probably less than in the external solution. If, however, hydrolysis of sucrose preceded absorption, the sugar gradient must still have been negative. But as nothing is known concerning the composition of the reducing sugars in the leaf, it is impossible, even if hydrolysis did precede absorption, to say whether sugar entered the cell with or against the gradient.

(b) *Oxygen* (*Experiment 4*).

In experiment 2 we noted that in the presence of oxygen starch formation proceeded more rapidly than when it was absent. The present experiment was designed with a view to learning if this also holds good for the uptake of sugar by the cell. The method of procedure was similar to that of the last experiment, except that one group of discs was kept in an oxygen-free atmosphere (nitrogen) as in experiment 2, while the other was in air. Both groups were exposed to light for twelve hours. The concentration of sucrose used was again 0·1 per cent.

The results are shown in Table III. The dry weights of the Air and Nitrogen groups are both less than the Initial, while that of the Air is less than that of the Nitrogen. The sampling of these two groups is, however, very poor, and there is clearly no significant difference between them.

TABLE III

Weights of Sugar, etc., in Air and Nitrogen Groups

	Gm.		Mg.		Gm. per 100 gm. water.	
Treatment.	Water.	Dry weight.	Reducing sugars.	Sucrose.	Reducing sugars.	Sucrose.
Initial *a*	2·55	0·663	7·77	0·59	0·305	0·023
b	2·51	0·645	7·65	0·59	0·305	0·024
Mean	*2·53*	*0·654*	*7·71*	*0·59*	*0·305*	*0·024*
Air *a*	4·04	0·582	13·00	1·25	0·322	0·031
b	3·91	0·525	11·60	1·70	0·297	0·043
Mean	*3·98*	*0·554*	*12·30*	*1·48*	*0·310*	*0·037*
Nitrogen *a*	3·75	0·600	8·68	1·07	0·231	0·029
b	4·02	0·535	8·96	1·39	0·223	0·035
Mean	*3·89*	*0·568*	*8·82*	*1·23*	*0·227*	*0·032*

The weight of reducing sugars was, however, consistently much greater in the Air than in the Nitrogen group. For sucrose the Air group also exceeded the Nitrogen, but the difference was small and not significant. The concentration of reducing sugars was much greater in Air than in Nitrogen, while the sucrose concentrations were much the same in both groups. It would thus appear that, like starch formation, the absorption of sugar is hastened by the presence of oxygen. It is noteworthy that the concentration of sucrose was much less in the leaf than in the external solution. This conclusion remains unaffected by the corrections referred to in experiment 3. Thus if sugar entered the cell without preliminary hydrolysis it entered with the gradient.

IV. DISCUSSION

We have shown that diffuse daylight of very low intensity and oxygen, independently of one another, promote starch formation in previously destarched discs of leaf when immersed in dilute solutions of sucrose. We have also shown that this effect of light and oxygen on starch formation is probably due to the effect of these factors in hastening the uptake of sugar.

It has long been thought that light increases the permeability of the plasma membrane. Lepeschkin (1930) concluded that an increase in permeability under the influence of light is a general property of protoplasm. Very recently Ingold (1936) has shown that light increases the rate of absorption of salts by Elodea, but suggests that the effect occurs through the medium of photosynthesis rather than directly through a change in permeability. The effect of oxygen on salt uptake has been discussed in a series of important papers by Steward (1930, 1933, 1935), who concludes that uptake is related to respiration. In a recent paper (Phillis and Mason, 1936) we have pointed out that different tissues behave as if they had different solvent capacities for sucrose, and have suggested that solvent capacity, like the accumulating capacity of Steward, depends on some special organization of the cytoplasm, which for its maintenance requires a continuous supply of metabolic energy.

It would thus appear that the increase in the rate of sugar uptake by the leaf in light may be due to increased permeability, and the further increase in the rate of uptake in the presence of oxygen may be due to an increase in the solvent capacity of the cytoplasm. The actual state of affairs, however, may not be as simple as this, for light may influence the solvent capacity of the cytoplasm as well as the permeability of the membrane (cf. Brauner and Brauner, 1936), and oxygen may affect the permeability of the membrane as well as the solvent capacity of the cytoplasm. Moreover, the possibility cannot be dismissed that both light and oxygen affect the diffusion constant (cf. Mason and Maskell, 1928; Mason and Phillis, 1936*a*) of sugar in the cytoplasm.

V. SUMMARY

1. The formation of starch by discs of cotton leaf, previously destarched and then immersed in sugar solution, is accelerated independently by oxygen and by light.

2. Both factors would appear to hasten starch formation by increasing the rate of absorption of sugar by the leaf rather than by an effect on the starch–sugar equilibrium.

3. The possible mechanisms by which such increased absorption of sugar could be brought about are briefly discussed; though the data are insufficient to enable a decision to be made concerning the manner in which light and oxygen act, it seems probable that light increases the permeability of the plasma membrane and that oxygen affects the solvent capacity of the cytoplasm.

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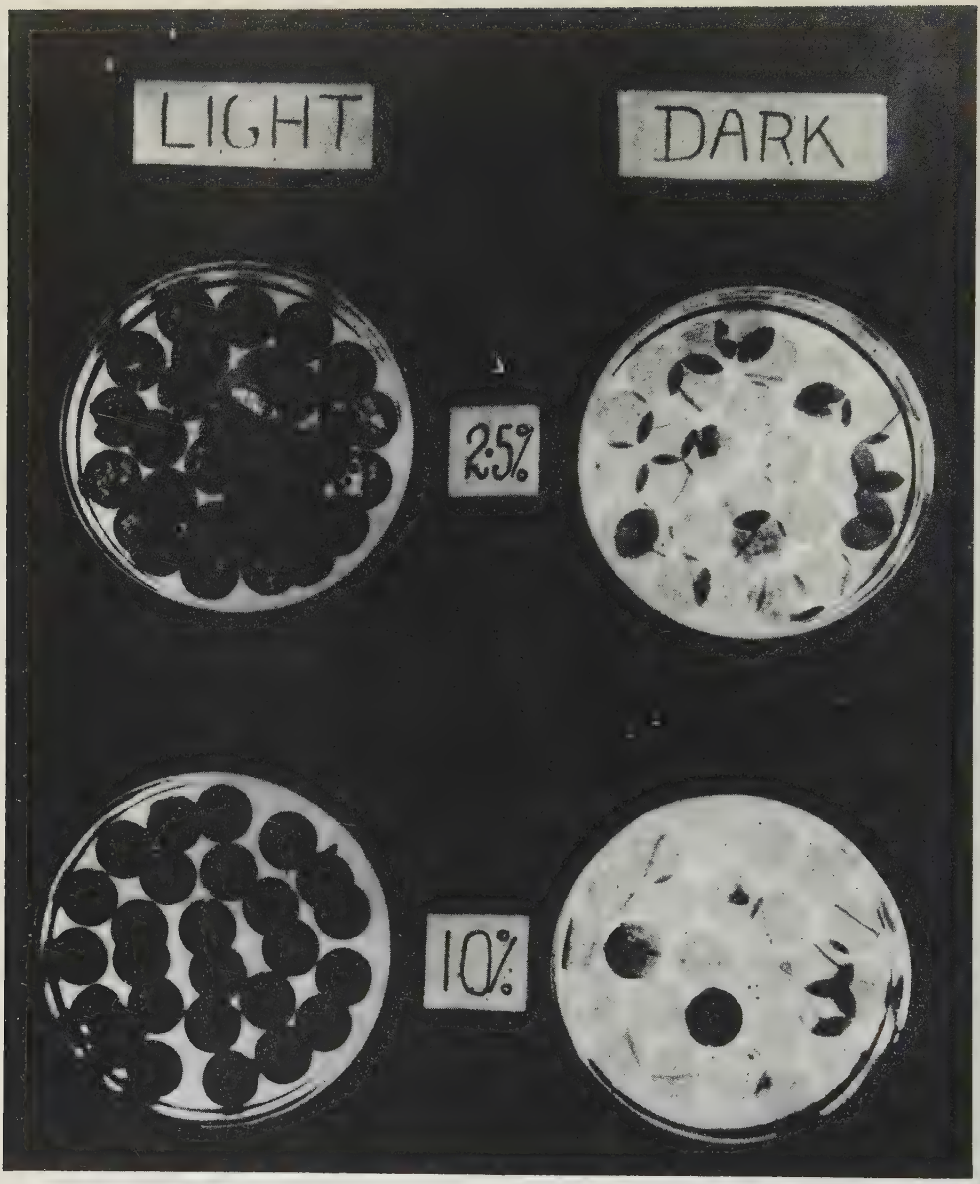
EXPLANATION OF PLATE IX

Illustrating Mr. E. Phillis's and Dr. T. G. Mason's paper on 'The Effects of Light and of Oxygen on the Uptake of Sugar by the Foliage Leaf'.

Fig. 1. The effect of light on the formation of starch in leaves immersed in sucrose solutions.

Fig. 2. The effects of light and of oxygen on the formation of starch in leaves immersed in sucrose solutions.



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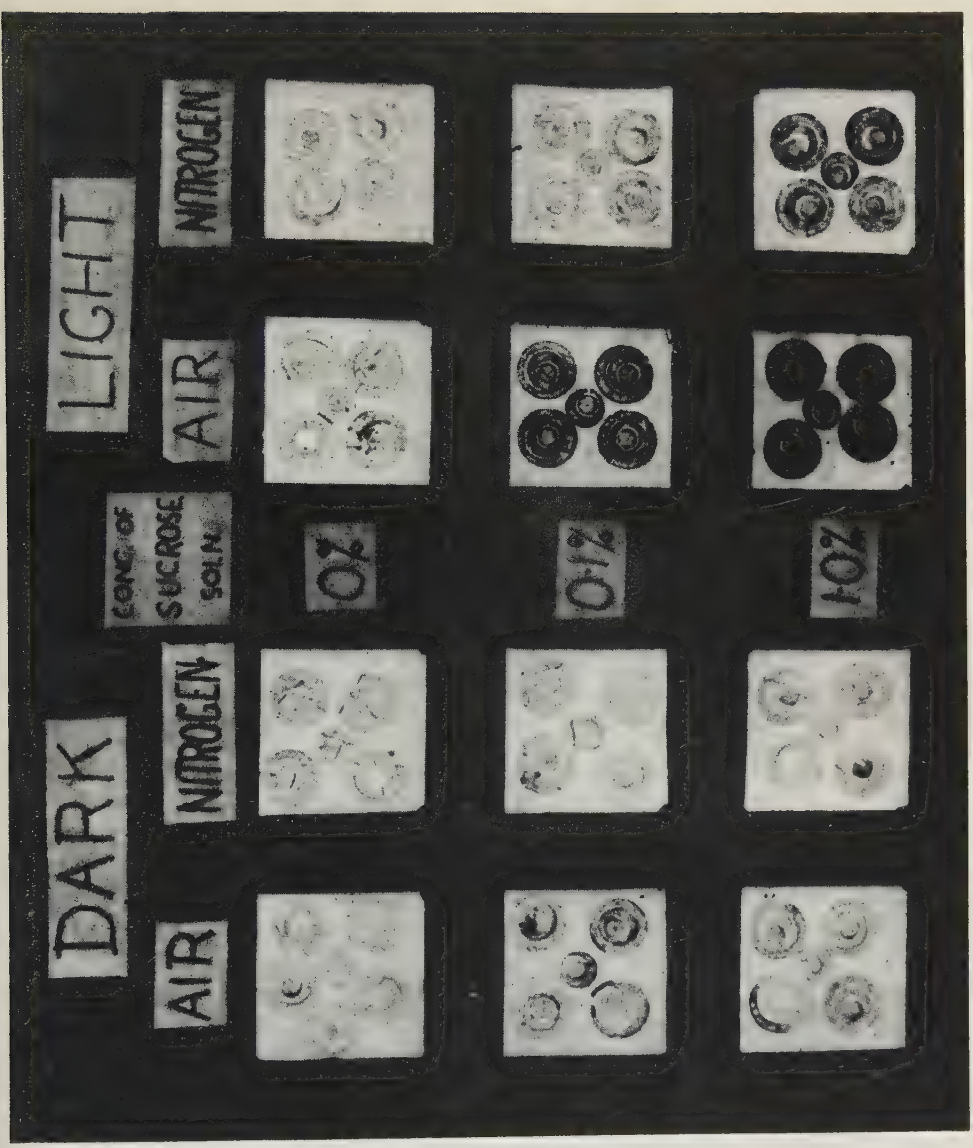
PHILLIS AND MASON – LIGHT AND OXYGEN AND THE UPTAKE



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AR.

Huth, Stubbs & Kent

The Method of Germination of Seeds Enclosed in a Stony Endocarp. II

BY

ARTHUR W. HILL, K.C.M.G., F.R.S.

With fourteen Figures in the Text

SINCE writing the account of the method of germination of certain seeds enclosed in a stony endocarp (Hill, 1933) a few other interesting cases have come to notice among seeds sent to Kew, as well as among some that are well known.

These include the Coconut Palm, *Cocos nucifera* Linn., the Doum Palm, *Hyphaene thebaica* (Linn.) Mart., *Jubaeopsis caffra* Becc., from South Africa, and its near relative the Chilian Palm, *Jubaea spectabilis* H.B.K., Balanites (Zygophyllaceae), Allospondias, Poupartia, Antrocaryon, and Choerospondias (Anacardiaceae), various species of the genus Parinari (Rosaceae), Panda (Pandaceae), Argania and Sideroxylon (Sapotaceae), Thevetia (Apocynaceae), and *Owenia venosa* (Meliaceae).

In connexion with the germination of the Doum Palm, the germ pore of *Cocos nucifera* has also been examined, since the thin protective covering over the pore is comparable to that of Hyphaene, and there appears to be no exact description or figure of the pore and its cover in the literature on the Coconut Palm.

All these four palms are of interest since the seed in each of them is enclosed in a thick shell or endocarp, but the germ pore—a very small area compared to the size of the 'seed'—is comparatively poorly protected. This pore or 'soft eye' in the Coconut is situated at the base of the nut; in the Doum Palm (*Hyphaene thebaica*), it occurs at the apex, while in Jubaea and Jubaeopsis, the three germ pores—one to each carpel—are situated in about the middle of the nut at its greatest diameter.

Of the three 'eyes' at the base of the Coconut (Sampson, 1923)—one to each carpel—only one, the soft eye, is functional, for under it is to be found the embryo, which gets carried through the pore when the cotyledonary tube or sheath, with the included embryo, presses against the soft eye on the commencement of germination, and forces off the lid covering the orifice. As in Hyphaene, the fibre—mesocarp—covering the nut is finer and more open over the soft eye than elsewhere. The 'soft eye' of the Coconut is more or less circular in outline, about 1·6 cm. in diameter, with a total thickness of about 0·8 mm. This cover or lid consists of two portions, the outer a thin shell, 0·2 mm. in thickness, representing a very thin portion of the

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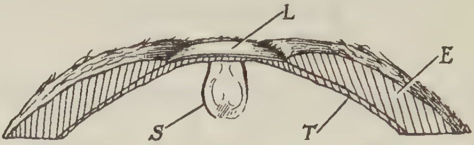
endocarp, underlaid by an inner layer, about 0·6 mm. thick, which is part of the testa. The lid fits tightly into the orifice in the thick endocarp of the seed, which is some 4 mm. in thickness, but thins out in the neighbourhood of the soft eye.



'FIG. 1. *Cocos nucifera* Linn., showing the outer thin shell (L), which is part of the endocarp, with the underlying portion of the testa (T) to which the embryo (S) is attached. A portion of the thick endocarp (E) surrounding the 'soft eye' is seen partly in section and partly in surface view (*nat. size*).

In *Hyphaene thebaica* Mart. (Palmaceae—Borasseae) the fruits are more or less globose, some 5 cm. in diameter. The shell or endocarp is covered by a thick layer of fleshy and fibrous mesocarp protected by an outer shining exocarp. At the apex of the fruit the fibrous tissue is less matted and can be easily disintegrated just above the apex of the woody endocarp. On removing the fibres, a small circular cap can be seen at the apex of the endocarp, which is pushed up—on the germination of the embryo—by the radicle enclosed in the cotyledonary sheath, thus allowing the emergence of the radicle and later of the plumule through the orifice in the endocarp.

The cap, which is almost circular in outline, 4·75 mm. long by 5 mm. broad externally, is about 0·37 mm. in thickness, domed and polished on the inside; internally it measures 4 mm. and 4·5 mm. in diameter in length and breadth. When attached to the endocarp, to which it is hermetically sealed, though it is a perfectly separate structure, the cap and endocarp wall appear to be continuous and hardly any trace of the existence of the beautifully fitting little cap can be detected (Fig. 2).

The nut of *Jubaeopsis caffra* is furnished with three pores situated midway on the sides of the nut. They are usually more or less circular in outline, but they may be somewhat heart-shaped and either rounded or rather pointed above. The pores are closed by a 'lid' or diaphragm of thinner lignified tissue, which stretches across the orifice and is 'flush' with the outer surface of the nut.

The seed inside the shell is covered by a thin, brown, membranous or cartilaginous testa and, as germination commences, the embryo enlarges in its sheath and the 'radicle' end of the sheath grows out towards the orifice as a pointed conical protrusion capped with the conical, cup-like covering of the testa. This cone breaks through the lid, which, however, is not pushed off in one piece, as in some palms, but is broken through and the tip of the sheath then pushes itself through the orifice. The growth of the sheath tip causes the testa to rupture with the result that a small conical cap is left on the tip of the sheath, which is pushed out and can be seen lying on the side of the sheath as it emerges (Fig. 3). As in Phoenix and other palms, the sheath tip bends vertically downwards as soon as it emerges from the orifice and grows downwards carrying the contained plumule and radicle some depth into the soil, the first leaf eventually breaking through the tubular sheath and expanding above ground.

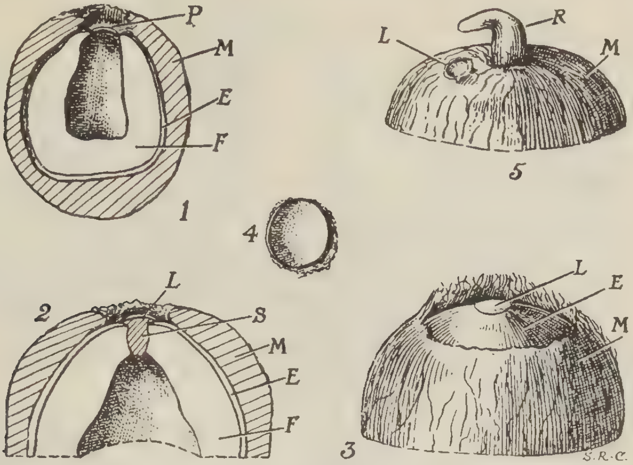


FIG. 2. *Hyphaene thebaica* Mart. 1. The nut in section showing the fleshy and fibrous mesocarp (M), the thin woody endocarp (E), the circular pore (P), and the endosperm (F) enclosed in the testa (× ⅘). 2. Another section showing the embryo (S) lying under the cap (L) at the apex of the fruit where the mesocarp has thinned out (× ⅘). 3. A surface view of the fruit-apex to show the thinned-out mesocarp (M) exposing the apex of the endocarp (E) and the lid (L) (× ⅘). 4. The lid detached, seen from the inside (× 2). 5. The germinating fruit with the sheath (R) emerging: the pushed-off lid (L) is seen lying on the surface of the mesocarp (M) (× ⅘).

The nut of Jubaea is very similar to that of Jubaeopsis with similar lateral germ pores. On germination the lid, unlike that of Jubaeopsis, is pushed off whole and thus allows the germ tube to grow out through the opened pore. The lid is circular or ovoid, with a polished inner surface. On emerging from the nut the sheath pushes through the testa, part of which may be seen surrounding the base of the germ tube (Fig. 4).

The germination of *Elephantorrhiza Burchellii* Benth. (Leguminosae) may be mentioned here as the method is comparable to that of Phoenix, Jubaeopsis, and other palms (Hofmeyer, 1921). The embryo is carried out of the seed on germination in a cotyledonary tube and buried deep in the ground, the cotyledons remaining in the seed. The plumule later bursts through the tube as in Phoenix and comes above ground.[1]

Turning now to examples among the Dicotyledons, which have not been previously described, it will be more convenient to deal with them according to the devices they exhibit, rather than to arrange the various genera and their families in proper systematic order.

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[1] Cf. also the germination of *Megarrhiza californica* (Hill, 1916).

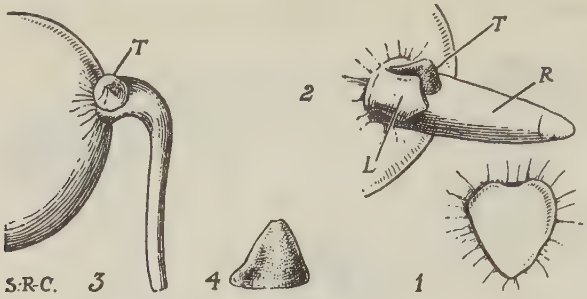

FIG. 3. *Jubaeopsis caffra* Beccari. 1. The cordate lid (×2). 2. The emergence of the germ tube or sheath (R) containing the embryo, showing the fractured lid (L) and the cap of the testa (T) pushed off from the tip of the sheath (*nat. size*). 3. The germ tube growing down with the testa cap (T) by the side of the orifice (*nat. size*). 4. The testa cap (×2).

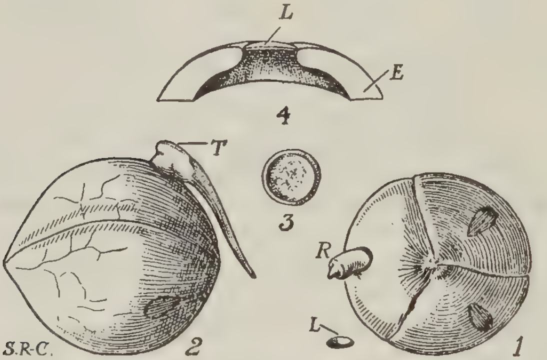

FIG. 4. *Jubaea spectabilis* H. B. K. 1. The nut seen from above with its three ridges and three germ pores, from one of which the germ tube (R) is emerging: (L) the pushed-off lid (*nat. size*). 2. The germinating nut to show the germ tube pushing through the testa (T) (*nat. size*). 3. The lid (×2). 4. One of the pores in section to show the thickness of the endocarp wall (E) and the lid (L) covering the germ pore (×2).

Sideroxylon (Sapotaceae) may first be mentioned, though the stony endocarp does not appear to be specially modified for liberating the seed.

On germination the endocarp separates into four or five irregularly shaped pieces and, though, no doubt, the fractures occur along definite planes of weakness, formed during its development, the resultant fragments have no definite shape and there is no pushing aside of a particular lid or flange, but the germinating embryo escapes by the general fragmentation of the whole of the endocarp covering.

The other cases to be described, however, have evolved very definite devices, both for protecting the seeds and also for allowing the seedling to escape.

Dealing first with those endocarps which split off a portion of the stony wall in the form of a flange or shutter after the manner of Cornus, Tectona, &c., described in the previous paper, Balanites (Zygophyllaceae) may first be considered. The ovary in this genus is 5-locular, but the ripe fruit contains only one loculus with a single seed. The endocarp, which is enclosed in the oily mesocarp, at the commencement of germination develops five equidistant splits from the apex to about the middle of the stone, thus separating the apical half of the stone into five equal triangular portions. As the embryo

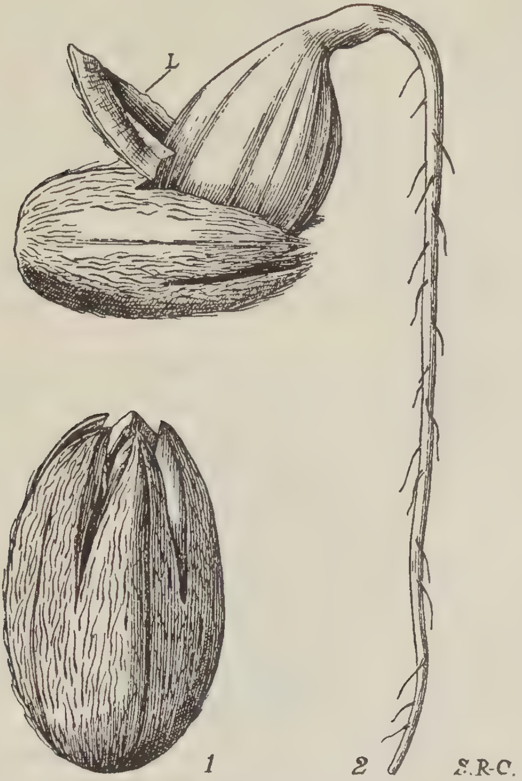

Fig. 5.

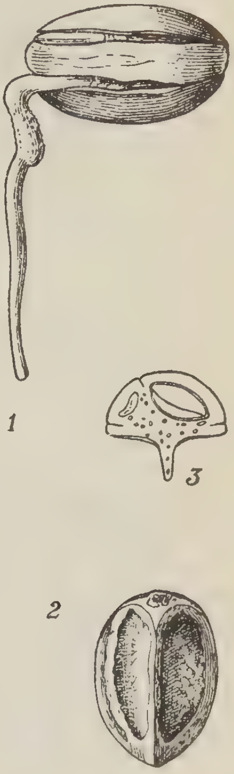

Fig. 6.

Figs. 5 and 6. Fig. 5. 1. *Balanites Roxburghii* Planch.; the stone at the commencement of germination with the endocarp split at the apex into five equal triangular segments (*nat. size*). 2. *B. aegyptiaca* Del.; the seed germinating and emerging from the endocarp by pushing up one of the five flanges (L) (*nat. size*). Fig. 6. 1. *Argania Sideroxylon* Roem. et Schultes; the endocarp stone showing one seedling germinating and the upper one still covered by the shutter-like valve, the outer wall of the cavity. 2. A stone showing two empty cavities with the shutters thrown off. 3. The same in transverse section with two empty cavities below and one immature one above, and one containing a seed showing the limits of the detachable valve or shutter.

develops and the radicle begins to emerge, one of the triangular flanges is pushed up at right angles to the basal portion of the endocarp, after the manner of the device in Canarium (Hill, 1933, fig. 6). The triangular flanges, which measure about 2·3 cm. long and 1·5 cm. broad at the base, are 3 mm. thick, and this is the thickness of the endocarp generally. Only one of the five flanges into which the endocarp splits appears to be hinged, so that it can be pushed up like a flap by the emerging embryo. There is no sort of indication on the outer surface of the endocarp at the base of any of the flanges to show which of them will be the one to be pushed up on germination. It may be, therefore, that the pressure of the developing embryo is sufficient to cause the fracture at the base of the flange and that any one of the five can be so broken, the governing factor being that it is the particular flange which is pressed against by the emerging radicle which is the one to be pushed up. The two species studied, *B. aegyptiaca* and *B. Roxburghii*, are essentially similar in the structure of the endocarp and the mode of liberation of the germinating seed.

The genus Argania (Sapotaceae), represented by *A. Sideroxylon* Roem. et Schult., a native of Morocco, affords an interesting example of the 'shutter' device, somewhat similar to that exhibited by Tectona (Hill, 1933, fig. 7). The fruit of Argania contains an ovoid stone, enclosing four loculi which run the whole length of the 'stone'; as a rule, however, only two seeds come to maturity.

On germination the outer portion of each loculus separates off, like a curved, detachable shutter, and exposes the hollowed-out cavity of the endocarp in which the embryo has lain. This is very similar to the arrangement shown by Tectona, where the whole of the side of the wall covering the seed cavity becomes detached, and where also no part is left attached to the main portion of the endocarp, as is the case in Canarium and Davidia.

Panda oleosa Pierre (Pandaceae), from the Gold Coast, is another interesting example of the 'shutter' device of rather a striking nature (Hutchinson, 1926). Its large globular fruits are fleshy, 5–6 cm. in diameter, and contain a hard, stony, turbinate endocarp, 5 cm. long by 3·5 cm. across, enclosing three, or sometimes four seeds. The seed cavities in transverse section are V-shaped or almost semicircular, somewhat constricted in the middle, and are arranged with their concavities towards the centre of the stone. The thickness of the stone varies from 1·25 cm. at the centre of the seed to 5 mm. at the two extremities. Externally the hard stone, which is pointed at the apex, is uniformly pitted with small depressions and resembles in appearance a lump of spongy rubber. Three, rarely four, slightly marked ribs run the length of the stone, and between them indications can be seen of the outlines of the large flanges which separate off when the seeds germinate.

Each flange or valve is cordate or shield-shaped in outline, 5 mm. thick at the edges and 1·25 cm. at the centre, where it projects into and narrows the seed cavity. The inner face of the flange forming the outer surface of the seed cavity is smooth and highly polished, as are also the inner surfaces of the cavities, which—with the stout ribs—form the triangular (or occasionally quadrangular) woody structure of the endocarp, which remains when the flanges are thrown off on the germination of the seeds. These cavities are naturally deeply concave and cordate in outline with a strongly marked, V-shaped, apical depression.

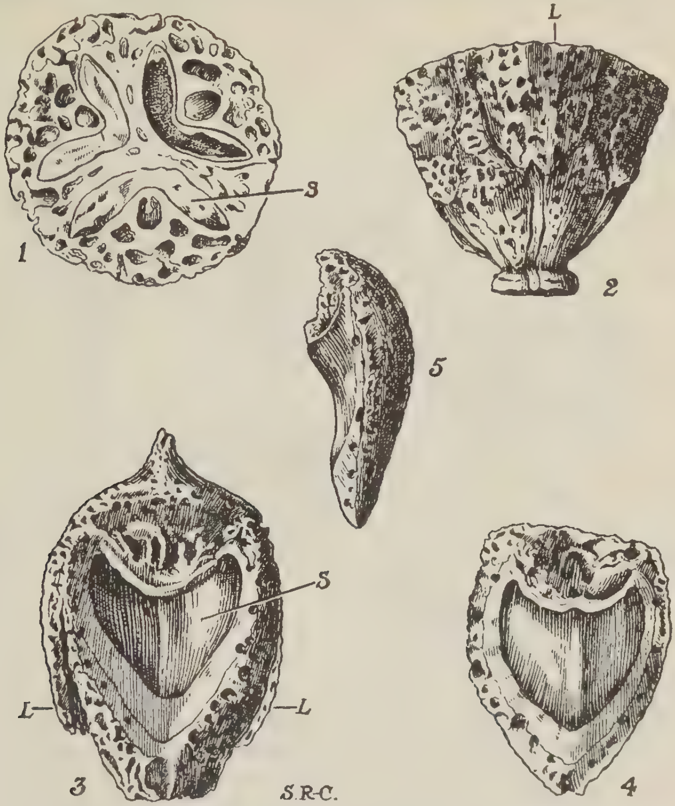

FIG. 7. *Panda oleosa* Pierre. 1. The stone in transverse section to show the three V-shaped cavities which contain the seeds (S) enclosed in the hard, sponge-like endocarp (*nat. size*). 2. Surface view of the lower part of the stone; the outlines of three of the shutters or valves (L) can be seen (*nat. size*). 3. The stone with one valve removed to show the seed cavity (S); the outlines of two other valves (L) can be seen at the sides (*nat. size*). 4. A detached valve seen from the inside showing the highly polished inner surface marking the area of the seed cavity (*nat. size*). 5. The same in side view to show the thickness of the valve and its projecting median ridge (*nat. size*).

The mechanism resembles that of Canarium, Cornus, and Tectona (Hill, 1933, figs. 5, 6, and 7), but in the case of Panda the valve or flange has a pointed base and more closely resembles the valve of Tectona, since its basal portion is not cut off by a straight transverse line as in Canarium and Cornus.

A more complicated state of affairs is exhibited by *Thevetia peruviana* (Pers.) Merrill (*T. nereifolia* Juss. ex Steud., Apocynaceae). This plant, a native of central and southern tropical America, also bears oil-yielding fruits. The somewhat flattened turbinate drupe of Thevetia encloses the thick-walled stony endocarp, which is more or less bluntly triangular in outline or almost cordate, from 1·7–2·3 cm. long to 3–3·3 cm. broad, and about 1·8 cm. across, oval in cross-section. The stone is marked by deep grooves which cut it into four equal triangular quadrants, each of which contains a single seed surrounded by its testa. On germination the endocarp splits, not by the groove across the middle or broader portion, but by the more pronounced fissure which circumscribes the endocarp along its longer axis, and is particularly marked as a broad groove running along the flat top of the stone (Fig. 8, nos. 1 and 2). The stone thus splits into two equal halves and not into

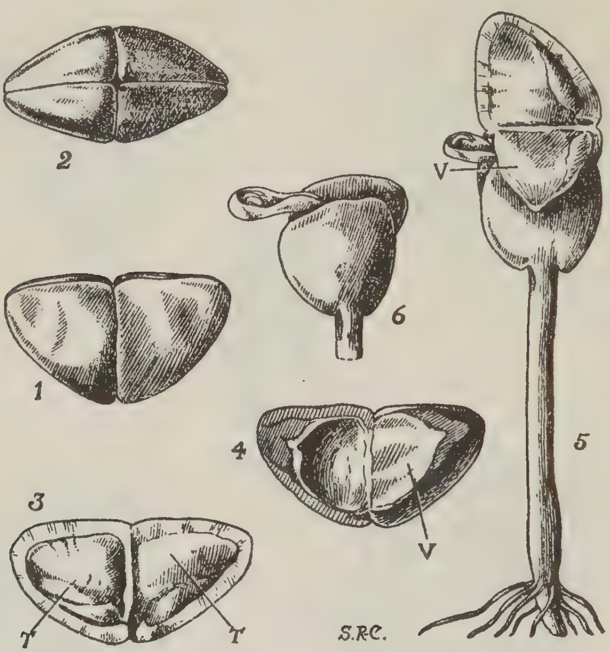

FIG. 8. *Thevetia peruviana* (Pers.) Merrill. 1. The endocarp in side view showing the deep lateral and apical grooves (*nat. size*). 2. The same from above to show the four quadrants of the stone (*nat. size*). 3 and 4. The stone split into its two halves; each quadrant may contain a seed (*nat. size*). In Fig. 3, two seeds can be seen enclosed in their testas (T); in Fig. 4 the seed is empty and one valve (V) of the testa has been pushed back (*nat. size*). 5. The germinating seedling emerging from the testa with the cotyledons still held by the testa valves (V) (*nat. size*). 6. The cotyledons removed from the seed to show the way in which the plumule extricates itself from the seed cavity (*nat. size*).

four portions as might be expected, and this opens the way for the subsequent germination of the seeds. There may be as many as four mature seeds, one in each loculus, but usually only two seeds, one in each half of the stone, come to maturity (Fig. 8, nos. 3 and 4).

The splitting of the endocarp, however, is only the first stage in germination, for each embryo is further enclosed in a somewhat woody or coriaceous testa. The seed itself is roughly triangular in outline, with the side attached to the vertical median line of the endocarp straight, the other edges being slightly curved, following the shape of the endocarp cavity. The method of dehiscence of the seed may be compared to the opening of an oyster, since the testa when ripe becomes separable into two halves or valves which are firmly united along their edges and hinged together along the straight vertical axis (Fig. 8 nos. 3, 4, 5).

On germination the swelling of the embryo forces the two valves apart and they separate to allow the emergence of the radicle and later of the cotyledons; the valves, however, do not break apart, but merely separate like the shells of an oyster—the embryo within representing the oyster itself—and remain hinged together along the whole length of the straight vertical edge.

The radicle becomes firmly fixed in the soil while the fleshy cotyledons are being held for some time between the valves of the testa, so that the plumule has to push its way sideways from between the cotyledons and the testa valves. Eventually, however, the cotyledons are pulled out from between the valves and emerge into the air.

The next cases to be described are those where the seed is secured in its cavity in the endocarp by a stopper or cork, as in Sclerocarya and Dracontomelon (Hill, 1933, figs. 9, 10). In the fruits of *Parinari macrophylla* Sabine (Rosaceae) from Northern Nigeria, and in almost all the other species of this genus, a close-fitting stopper or cork seals the orifice of the seed cavity. The seed kernels it may be noted contain useful oils.

The endocarp of the bilocular fruits is woody and corrugated and beaked at the base, but often only one of the two cavities contains a seed. On either side of the beak are two circular, pit-like depressions, which mark the positions of the cavities containing the seeds, and the orifice of each pit is filled by the lid-like stopper which plugs the basal end of the seed cavity. The appearance of the basal end of the stone with its two circular depressions separated by the beak is remarkably like the face of an owl. On germination the plugs or stoppers are forced out and ejected like corks from a bottle (Figs. 9 and 10).

The seed cavities, which are about 2·3 cm. long by 1 cm. broad, are lined with a fine, soft, brown wool, which is furnished by the fruit, and the inner surface of the plug is also covered by a similar woolly felt, so that the seed lies in a warm, woolly-lined box. The plugs or stoppers are circular, about 5 mm. in diameter on the outer side, and 3·5 mm. in diameter on the inner face.

This type of tapering stopper is similar to that of Sclerocarya and is found,

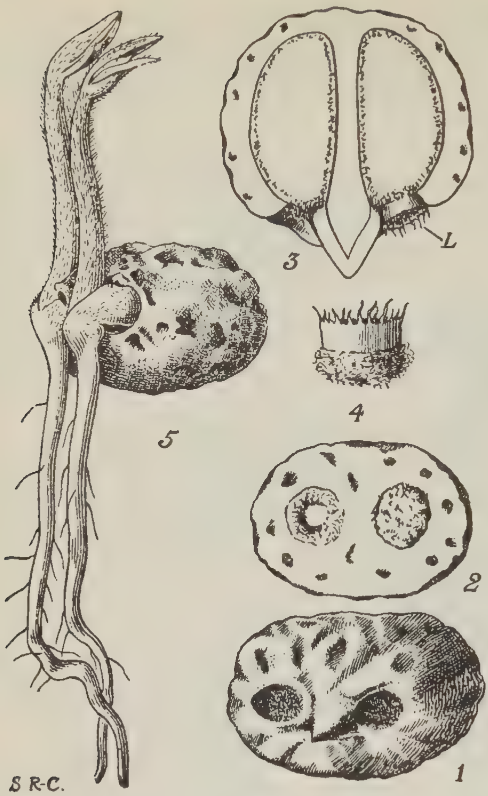

FIG. 9.

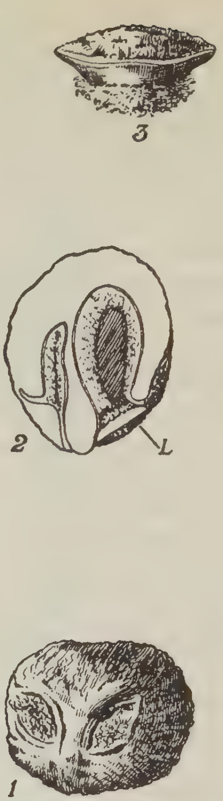

FIG. 10.

FIGS. 9 and 10. Fig. 9. *Parinari macrophylla* Sabine. 1. The stone with its basal beak and two germ pores sealed with stoppers and lids (*nat. size*). 2. The stone in transverse section to show the two seed cavities lined with fine wool (*nat. size*). 3. A longitudinal section showing the wool-lined seed cavities: in one the stopper (L) is in place, in the other it has been removed (*nat. size*). 4. The stopper detached, with its covering on the underside and stout hairs on the outer surface (× 2). 5. A germinating fruit with two seedlings: the cotyledons do not leave the seed cavities (*nat. size*). Fig. 10. *Parinari curatellifolia* Planch. ex Benth. 1. The stone with its basal beak and two germ pores (L) (*nat. size*). 2. The stone in longitudinal section, showing the wool-lined seed cavities, one of which is closed by the stopper (L) (*nat. size*). 3. The stopper (× 2).

with two exceptions,[1] in all other species of the genus. Other good examples are furnished by the tropical African *P. Mobola* and *P. curatellifolia.*

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[1] Two species of Parinari have been noticed which do not show this stopper-mechanism. *P. glaberrima* Hassk. (*P. laurina* A. Gray), from the Malayan region, has much larger fruits than is usual in the genus; they contain, however, only a single seed, which consists chiefly of the much convoluted, pink, fleshy cotyledons; the radicle splits the fruit open through the scar of the pedicel, but there is no pore or stopper.

The second aberrant species is *P. coriacea* Benth., a native of Brazil; when the flesh of the fruit is removed a smooth, hard stone is disclosed with an appressed beak on one side; this is quite unlike anything yet known in Parinari, and the species should probably be placed in a distinct genus. Dr. A. Ducke of Rio de Janeiro, to whom we wrote asking for fruits of this plant, replies that he also considers that it is not a true Parinari. Without further material, however, it is not possible to suggest the genus in which it should be placed.

The family Anacardiaceae, in particular the tribe Spondieae, affords many interesting examples of ingenious devices for seed protection and for the liberation of the seedling on germination, some of which have already been described (Hill, 1933).

In the genus Antrocaryon the stony portion of the fruit appears, at first sight, exactly to resemble the stony endocarp of Dracontomelon, since there are five lids visible on the surface of the stone, but it proves on closer examination to have a close affinity both with Pleiogynium (Hill, 1933, fig. 11) and with Choerospondias to be described later. In *Antrocaryon micraster* A. Chev. et Guillaumin, from tropical Africa,[1] the 'apple-shaped' fruit, depressed at the apex, consists of an outer skin, the exocarp, covering a fleshy mesocarp, surrounding the large, spherical, central stone, 3·3–3·5 cm. in diameter and 3 cm. high. In the centre of the circular or obscurely pentagonal stone five loculi may be seen in transverse section, arranged like a star with the loculi radiating from the centre to the circumference (Fig. 11). These loculi are lined by hard, shiny walls, which apparently represent the endocarp. The mesocarp, it is assumed, consists of two parts, an outer fleshy layer and an inner stone in which the stellate endocarp is embedded; the outside of the stone is marked by numerous pores, and in section it is seen to be riddled by small holes. The endocarp boxes are fused to one another and to the stony mesocarp at the base, but near the top of the stone they diverge outwards and their upper parts lie in cavities in the mesocarp: the actual apex of each endocarp 'box' is flattened and presses against the inside of a hard stony cap or lid in the mesocarp and when the cap is removed a fissure can be seen along the end of the endocarp showing where it splits to permit the escape of the radicle (Fig. 11, no. 3). The cap or lid, against which the tip of the endocarp box presses tightly, seals the opening in the mesocarp and the point of contact with the top of the endocarp leaves a clear scar on the inner surface of the lid.

The lids or caps are rounded on the outer side and more or less pointed on the side facing the centre of the stone, the larger ones being about 1·3 cm. long by 0·9 cm. broad and 3–4 mm. in thickness. The germination of *A. micraster* exactly resembles that of Sclerocarya, the growing radicle pushing up the lid, sealing the cavity as it emerges from its endocarp box. It is interesting to notice in how many genera of Anacardiaceae this lid device occurs—in some being an endocarp in others a mesocarp structure—and also to find that a similar arrangement has been evolved in the Rosaceae.

How far one may be correct in the use of the terms mesocarp and endocarp for these complicated stony fruit structures it is difficult to say, as the line of division between endocarp and mesocarp is not always clear. From what has been said, however, it would seem that in Dracontomelon the stone with

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[1] Some specimens of this plant from the Cameroons and Uganda have become known as *A. polyneurum* Mildbr., but a description of this plant has never been published. The specimens are indistinguishable from *A. micraster* and that species is now known from the Ivory Coast, Gold Coast, Southern Nigeria, Cameroons, and Uganda.

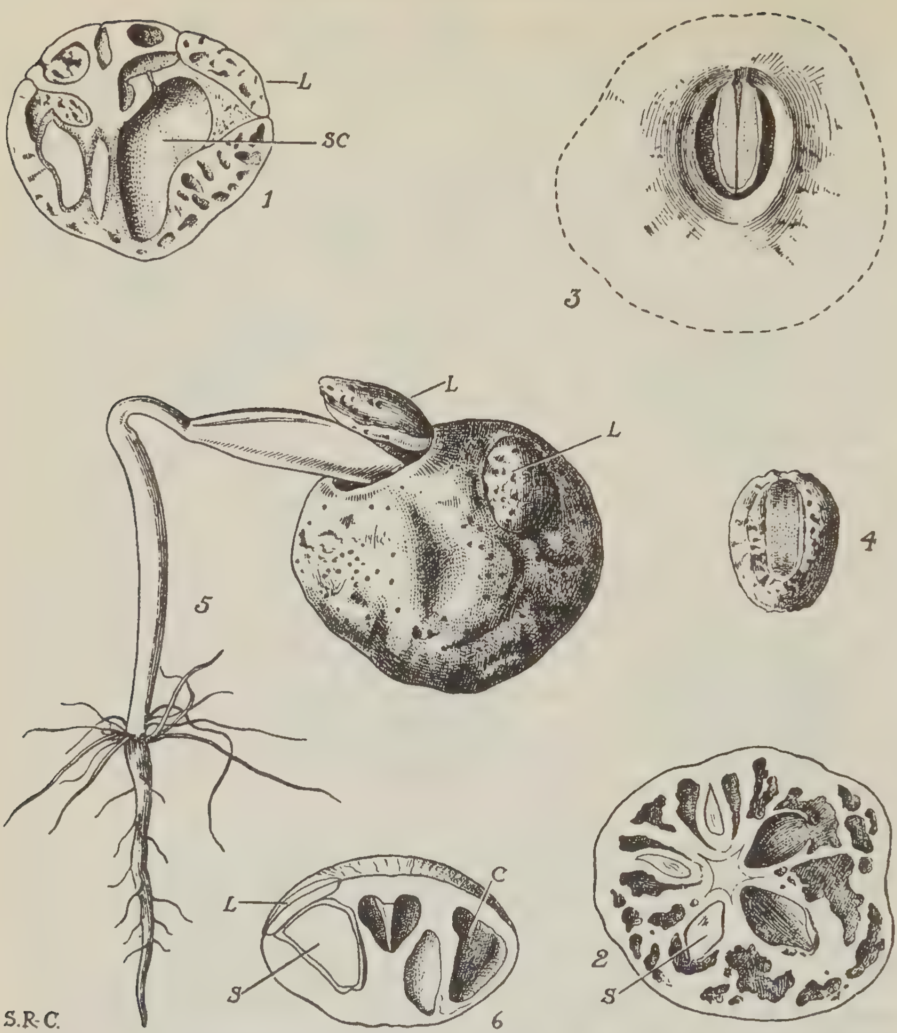

FIG. 11. 1. *Antrocaryon micraster* A. Chev. et Guillaumin; the stone in longitudinal section showing the seed cavity (SC) surrounded by its endocarp box embedded in the mesocarp and the lid (L) sealing the orifice in the mesocarp above the apex of the endocarp box (*nat. size*). 2. The same in transverse section showing the stellate arrangement of the seed-cavities (S) (*nat. size*). 3. The same, surface view of the stone showing one orifice with the lid removed and the thickened apex of the endocarp box with its median split (*nat. size*). 4. The same, the lid seen from the inside (*nat. size*). 5. The same, one of the seeds germinating and pushing up the lid (L) another lid (L) being in position (*nat. size*). 6. *A. amazonicum* (Ducke) B. L. Burtt et A. W. Hill, the stone in longitudinal section showing a seed (S) in a seed cavity and the lid (L) closing it; (C) is one of the cavities in the mesocarp which alternate with the fertile seed cavities (*nat. size*).

its lids should be regarded as endocarp, since the seeds in the cavities are covered only by their testas and not enclosed in any other structure, whereas in Antrocaryon, although the stone and lids appear at first sight strictly comparable to the similar structures in Dracontomelon, it seems to be more correct to assume that they are formed from the inner portion of the mesocarp. Whatever may be the true interpretation of these structures the interesting point emerges that, whereas in Dracontomelon the seeds themselves lie in cavities in the stone sealed by lids, in Antrocaryon the similar, sealed cavities contain the endocarp 'boxes' which further enclose and protect the seeds.

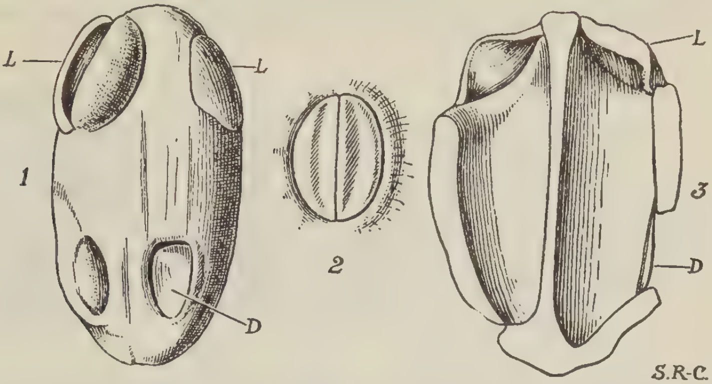

FIG. 12. *Pseudospondias microcarpa* (A. Rich.) Engl. 1. The stony endocarp showing the apical pores covered with lids (L) which are double structures like a pair of shutters, and the basal depressions (D) (× 3). 2. The lid in surface view showing the median line dividing it into two halves (× 3). 3. The endocarp in longitudinal section showing two seed cavities with half a lid (L) in position over each and one of the basal depressions (D) with its thin endocarp covering (× 3).

Another fruit of the same type is that of *Antrocaryon amazonicum*,[1] a native of Brazil, which previously has been placed in the genus Poupartia. The stone is more or less pentagonal in outline, about 3·5 cm. in diameter and 2 cm. high. The lids, closing the large seed cavities, are ovoid and somewhat pointed at either end, measuring about 1 cm. in length and 4·5–5 mm. in breadth. It has not been possible to study the actual germination of this species, as fresh fruits have been unobtainable, but in the structure of flower and fruit it shows a general agreement with the African species of Antrocaryon. The endocarp 'boxes', however, have very much thinner walls than in *A. micraster* and the stony part of the mesocarp is also thinner; large well-defined cavities alternate with the fertile loculi in contrast to the hard, sponge-like mesocarp tissue which is characteristic of *A. micraster* (Fig. 11, no. 6).

The genus Pseudospondias affords another interesting case which has been studied in the tropical African species, *P. microcarpa* Engl. This also resembles Dracontomelon to some extent in the nature of its fruit, since the cavities in the endocarp are closed by lids as in that genus and in Sclerocarya and Haematostaphis. The stony nut of *Pseudospondias microcarpa* is irregularly ovoid, from 1·5 to 1·8 cm. long and 0·9 to 1·1 cm. broad, 4-locular, with eight deep oblong paired depressions in the lower part of the stone and four large oval lids at its apex. The lids, which are rounded at each end, are bisected by a median longitudinal line running the length of the lid on a slight ridge on the outer surface. On germination the lid splits into two halves along this line, as in Haematostaphis (Hill, 1933, fig. 10). The lid, separating into its two halves, like the opening of a pair of shutters, thus allows the radicle to emerge followed by plumule and cotyledons.

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[1] *Antrocaryon amazonicum* (Ducke) B. L. Burtt et A. W. Hill, comb. nov. *Poupartia amazonica* Ducke in Archiv. Jard. Bot. Rio de Janeiro, iii. 204 (1922).

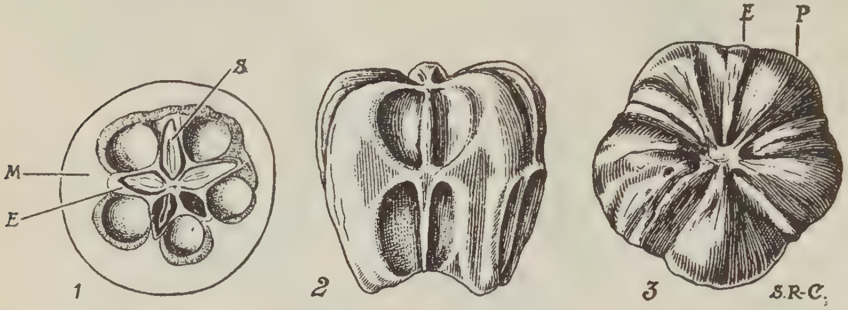

FIG. 13. *Allospondias lakonensis* (Pierre) Stapf. 1. The fruit in transverse section to show the fleshy mesocarp (M), the stellate endocarp (E), and the five circular patches of parenchymatous tissue lying between the rays of the endocarp-star and protected on the outside by strong fibres; seeds (S) may be seen in three of the endocarp cavities (× 3). 2. The fruit with fleshy mesocarp removed (× 3). 3. The fruit seen from above showing the five protruding parenchymatous bodies (P) with rounded outlines, separated by the five ridges (E) of the endocarp star (× 3).

The paired depressions on the stone of Pseudospondias, which are so conspicuous a feature, appear to be homologous with the peripheral depressions on the turbinate fruit of *Dracontomelon sinense* (Hill, 1933, fig. 8); or in those of *D. vitiense* Engl.—where they are much larger and are situated under the edge of the stone. The significance of these depressions has not been discovered.

In their fruit, or rather in the structure of the endocarp, the genera Pseudospondias and Dracontomelon show close resemblances. The stone of Pseudospondias may, in fact, be regarded as an elongated Dracontomelon.

Another interesting and peculiar fruit is that of Allospondias. The small ovoid fruits of *Allospondias lakonensis* (Pierre) Stapf,[1] which are about the size and shape of Sloes (*Prunus spinosa*), present a curious and remarkable structure when seen in transverse section. There is an outer fleshy layer enclosing a central star-shaped endocarp, and the angles between the five rays of the star are occupied by circular patches of white parenchymatous tissue, which in longitudinal section are seen to run the whole length of the fruit. The star-like, woody endocarp and the five parenchyma bodies are enclosed in a general lignified covering, which can be separated as a whole from the fleshy portion of the fruit. These white bodies, which Stapf refers to as 'cavities' in his description (Stapf, 1900), are actually masses of thin-

[1] This was also described as *Poupartia chinensis* Merrill in Phil. Journ. Sc., xv. 245 (1919).

walled, spongy parenchyma, the cells of which, when fresh, appear to be filled with mucilaginous material; but when dry the contents shrivel and there appear to be five cavities surrounding the star-shaped endocarp. The only other species of this genus, *A. laxiflora* Lace (Kew Bull. 1915, 397), has a similar 4–5 locular ovary, but only one or two of the seeds develop.

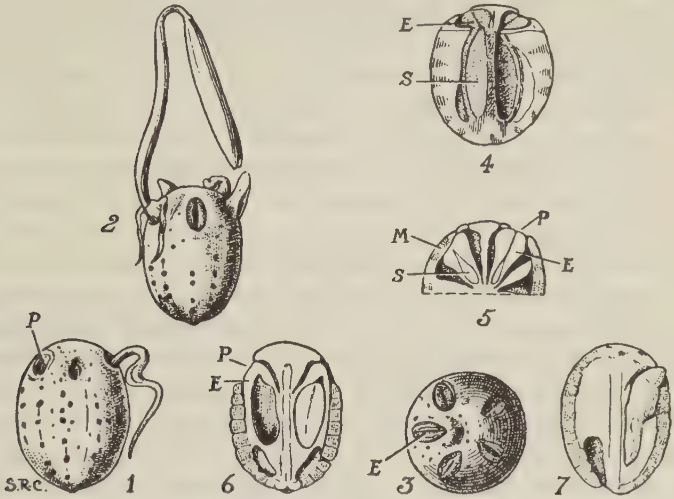

FIG. 14. *Choerospondias axillaris* (Roxb.) B. L. Burtt et A. W. Hill. 1. The ovoid stone showing the pores (P) below which lie the endocarp boxes; one of the seeds is germinating (*nat. size*). 2. Another stone with four seeds germinating (*nat. size*). 3. The stone seen from above to show the five pores; the membrane over one is still entire, but the others are broken and the tips of the endocarp boxes (E) with their longitudinal apical fissures are seen within (*nat. size*). 4. A stone cut longitudinally and across the pores, showing the seed (S) lying in its endocarp box (E) (*nat. size*). 5. A stone cut obliquely through a pore (P) to show the seed (S) enclosed in its endocarp box lying in a cavity in the mesocarp (M); the line of cleavage is clearly visible on the cut end of the endocarp box (E) (*nat. size*). 6. A four-seeded stone cut longitudinally through 2 pores (P) showing the endocarp boxes (E) (*nat. size*). 7. A germinating stone cut longitudinally (*nat. size*).

The sharply 5-ridged endocarp with the adjacent five parenchymatous bodies can be separated easily from the external edible pulp, while the parenchymatous bodies can in their turn be easily separated from the stellate endocarp, leaving this as a five-rayed structure, consisting of five narrow flanges or ridges, the actual endocarp containing the seeds.

The seeds of this interesting species, which were kindly sent by Professor H. H. Chung from Foochow in August 1936, have now germinated; the endocarp boxes split at the top like a Prunus stone and the two halves then separate to allow of the egress of the embryo. The seeds enclosed in their endocarp boxes in this respect show some similarity to those of Antrocaryon and to those of the next example, Choerospondias, which has been found to represent a new genus as the result of studying the fruit. Choerospondias has also been placed in the genus Poupartia and the species to be described is best known under the names *Poupartia Fordii* or *P. axillaris*.

Choerospondias axillaris[1] has a stony fruit which is also a double structure as in Antrocaryon and Allospondias. The seeds, enclosed in woody endocarp boxes, are sunk in cavities in the fibrous mesocarp which constitutes the ovoid stone. There is, however, no counterpart in this fruit to the five parenchymatous masses seen in Allospondias. The five thick-walled cylindrical or fusiform endocarp boxes containing the seeds of Choerospondias are much thickened at the apex. The apices are marked by a longitudinal slit or fissure, and it is along this fissure that the two halves of the endocarp box split and allow the radicle to grow out on germination. The endocarp boxes, as may be seen from the figures, are fused to the mesocarp towards their bases, but their upper portions lie free and unattached in the cavities of the ovoid stone, and are visible at the base of the five small oval pits situated around the top of the stone.

On germination the two halves of the endocarp box become separated by the pressure of the curved hypocotyl, and eventually the radicle emerges and frees itself from the endocarp and the stone. The arrangement here resembles that found in *Pleiogynium Solandri*, except that the inner boxes split at the apex in Choerospondias, while a cap—the upper portion of the endocarp—is thrown off from the apex of the box in Pleiogynium. The encircling 'stone', in both these genera, as well as in Antrocaryon, appears to be the mesocarp which has become woody. Choerospondias, unlike Antrocaryon, has no stony lids covering the orifices in the mesocarp; they are, however, closed, until germination takes place, by a thin membrane.

The only fruit so far met with which has at present baffled all attempts to discover the mode of escape of the imprisoned seeds is *Owenia venosa* F. Muell. (Meliaceae), from Australia. No one in its native country appears ever to have observed its germination. Fresh fruits have been sent over but their structure gives no clue as to how the strong stony endocarp can be opened, since neither pores nor flanges can be detected. The fruits have been placed in hot ashes, treated with sulphuric acid, filed, &c., and then sown in the most favourable conditions; germination, however, has not yet taken place, though more than a year has now passed since they were sown.

The following letter from Mr. W. W. Froggatt, formerly State Entomologist, New South Wales, who sent the seeds of *O. acidula*, is of interest in connexion with the difficulty of germinating these seeds:

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[1] **Choerospondias** *B. L. Burtt and A. W. Hill* (Anacardiaceae-Spondieae); genus novum *Spondiadi* et *Poupartiae* affine; ab illa petalis imbricatis, putamine osseo (nec fibroso), nervo intramarginali foliolorum deficiente, ab hac floribus, foemineis solitariis vel subsolitariis, masculis et pseudohermaphroditis paniculatis, numquam in racemos simplices spiciformes dispositis, fructu drupaceo putamine osseo ex endocarpio et mesocarpii parte interiore constante differt.

Species unica **Choerospondias axillaris** (*Roxb.*) *B. L. Burtt and A. W. Hill*, comb. nov. *Spondias axillaris* Roxb. *Poupartia Fordii* Hemsl. *P. axillaris* (Roxb.) King et Prain.

'Regarding the seeds of the "Emu Apple" or "Grurie" (*Owenia acidula*), I have not had any success with germinating the seeds up to date. I am, however, expecting some fresh seeds from Brewarrina shortly and am going to carry out further experiments.

'They must germinate from the seed, but the outer surface of the seed is as hard as ivory and the central germ not much bigger than an apple pip. It is hard to imagine that any germ could grow through such a hard covering. A western bushman tells me he has never seen a seedling and he thinks they send up suckers, and has promised to send me some.

'In my old bush days the emus used to eat the fruits and the seeds were passed through in their droppings; this treatment may act on the outer case. It is the only western tree seed that I have not been able to germinate.'

SUMMARY

A further series of devices exhibited by fruits whose seeds are enclosed in stony endocarps is given to supplement the account published earlier (Hill, 1933). The devices consist of simple splitting of the endocarp into two halves: caps or lids; cork-like stoppers or plugs, and shutters or flanges.

Further protection of the seeds is afforded by the mesocarp becoming stony as well as the endocarp. The structure of the fruit of Choerospondias, a new genus of Anacardiaceae, is described.

In order to facilitate references to these interesting cases of seeds enclosed in stony endocarps, which are found in so many different Natural Families, the various examples, described in the present and in the previous paper, are set out according to the particular kind of method that has been evolved for permitting the germinating embryo to escape from its protective stony enclosure.

I. Irregular splitting of the endocarp wall.
    Sideroxylon.

II. Simple splitting of the endocarp or endocarp loculi into two halves (at least at the apex).
  - (*a*) Endocarp unilocular splitting into two halves.
    Prunus, *Olea*, *Juglans.*
  - (*b*) Endocarp 4–5 locular, loculi splitting into halves at the apex, the endocarp further protected by a stony or fibrous mesocarp.
    Antrocaryon, *Choerospondias*, *Allospondias.*

III. Endocarp opening by throwing off shutters or flanges.
  - (*a*) One shutter only.
    Nyssa, *Mastixia* (fossil)—*Balanites* may be included here since one of the five valves into which the stone splits becomes bent back, as if hinged, to allow the emergence of the embryo.
  - (*b*) Two or more shutters.
    - (i) Those in which only part of the side of the seed cavity forms the shutter.
      Cornus, *Canarium*, *Davidia.*

(ii) Those in which the whole of the side of the seed cavity is thrown off on germination.
Panda, Tectona, Argania, Thevetia, Saccoglottis, Aubrya.

IV. Endocarp opening by throwing off lids or caps.
(*a*) Simple lids.
Cocos, Hyphaene, Jubaea, Jubaeopsis, Northea.
(*b*) Lids splitting into two halves on germination.
Haematostaphis, Pseudospondias.
(*c*) Corks or Stoppers—these are in the nature of stout, deep-fitting lids.
Parinari, Dracontomelon, Bertholettia, Lecythis (c.f. *Hippuris*).

In *Antrocaryon* the orifices in the mesocarp are sealed by similar plugs or stoppers (see II *b*). In *Pleiogynium* the endocarp is further protected by a stony mesocarp.

Method of germination unknown—*Owenia.*

My thanks are due to Professor H. H. Chung, Wu-Han University, Wuchang, for the fruits of *Allospondias lakonensis*; to Mr. H. Green, Superintendent, Botanic Gardens, Hong Kong, for the fruits of *Choerospondias axillaris*; to Dr. J. W. Bews, Professor R. H. Compton, and Dr. I. B. Pole Evans for those of *Jubaeopsis caffra*; to the Director of the Botanic Garden, Coimbra, for the fruits of *Jubaea spectabilis*, and to Mr. W. B. Cotton, who kindly sent the fruits of Hyphaene; to the Director of Forests, Nigeria, for fruits of *Parinari macrophylla*, and to Mr. R. E. P. Dwyer, Department of Agriculture, New Guinea, for those of *P. laurina.*

Mr. C. C. Calder, Superintendent of the Royal Botanic Gardens, Calcutta, kindly sent the fruits of *Balanites Roxburghii*, and Mr. Robbie, Government Garden, Khartoum, those of *B. aegyptiaca.*

For the fruits of *Antrocaryon micraster* I am indebted to Mr. W. D. MacGregor of the Forestry Department, Nigeria, and for those of *Thevetia nereifolia* to the Director of Agriculture, the Gold Coast. Mr. H. J. Solomon and Mr. Charles Binnie kindly sent the fruits of *Owenia acidula* from Australia.

My thanks are also due to Miss Ross-Craig for the series of drawings illustrating this paper, and to Mr. B. L. Burtt for his assistance, especially in connexion with the plants placed at one time under the genus Poupartia.

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Chromosome Studies in Hyacinthus orientalis L.

II. The Meiotic Chromosomes

BY

S. P. NAITHANI

With Plates X to XII and one Figure in the Text

THE spiral structure of the meiotic chromosomes has long been recognized. Baranetzky (1880) was the first to find spiral chromosomes in the pollen mother-cell of *Tradescantia virginica*. They have since been described in a considerable number of plants by various recent investigators, but there is still a considerable difference of opinion in their interpretation. Kuwada and other Japanese workers describe the first metaphase chromosomes as 'doubly coiled'. Darlington (1935) in *Fritillaria* and Sax (1935) in *Rhoeo discolor* observe the same structure. Nebel (1932*a*), Koshy (1934), Hoare (1934), Huskins and Smith (1935), and other workers describe them as singly coiled in their material.

MATERIAL AND METHODS

Bulbs of different varieties of *Hyacinthus orientalis* were obtained from Alexander & Brown, The Scottish Seed House, Perth. Some were obtained locally. The following varieties were examined:

1. *Gertrude* (diploid).
2. *Garibaldi* (diploid).
3. *La Victoire* (diploid).
4. *Yellow Hammer* (diploid).
5. *Lady Derby* (triploid).
6. *King of the Blues* (triploid).
7. *Queen of the Pinks* (triploid).
8. *L'Innocence* ($2n = 27$).
9. *City of Haarlem* ($2n = 23$).
10. *Captain Byoton* ($2n = 22$).
11. *Nimrod* ($2n = 19$).

Some of the bulbs were specially prepared by subjecting them to higher temperature for early flowering (see Part III of this series). Reduction divisions in pollen mother-cells started earlier in these specially treated bulbs than in the untreated ones, the former at about the beginning and the latter at the end of October. One of the varieties of the latter group, the *Queen of the Pinks*, however, did not start division until as late as the middle of December. Divisions in the pollen grains were correspondingly hastened, the treated bulbs showing them at the beginning of November and the untreated ones at the end of it.

The pollen mother-cells were smeared and fixed in La Cour's (1931) 2 BE and 2 BD, Taylor's (1924) fluid, medium Flemming, Benda's fluid, Smith's

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(1935) S_1 and S_2, and Belling's modification of Navashin's fluid. The study of early prophase stages was made both from smears and from paraffin sections. The anthers were dipped for a few seconds in Carnoy's fluid before putting them in the fixatives. It appears that the nucleus in Hyacinthus is less resistant to the action of fixatives than normally is the case with other plants. The chromosomes are contracted so much, especially between pachytene and diakinesis, that their structure at diplotene becomes obscured. A similar effect was reported by Darlington (1929) in studying the meiotic division in Hyacinthus.

Recent advances in cytology have revealed the fact that ordinary methods do not satisfactorily display the internal structure of chromosomes. Many investigators have found that various pretreatments bring out more details of chromosome structure. Kuwada and Nakamura's (1934) method, in which the pollen mother-cells were exposed to ammonia vapour for a few seconds and then stained in Belling's iron-aceto-carmine, was largely used. Preparations made in this way were then made permanent by McClintock's (1929) method. The spiral structure of the chromosomes was clearly revealed by this method.

Preparations were also made by La Cour's (1935) method. Since some difficulty was encountered in keeping the smears on the slide, the following alteration proved very successful. The slide was first dipped in 3 per cent. sugar solution. It was shaken well to keep as little sugar solution on the slide as possible; anthers were then smeared and exposed to ammonia vapour for a few seconds and immediately placed in the fixing fluids. Fixation was complete after two hours. Medium Flemming and La Cour's 2 BE proved better than other fixatives. Sakamura's (1927) hot-water method and Sax and Humphrey's (1935) pretreatment in 20 per cent. alcohol did not prove satisfactory, as the pollen mother-cells were washed off.

In place of ammonia the various acid vapours recommended by La Cour (1935) were also tried, but they did not give so satisfactory a result. La Cour gives a slight preference to nitric acid.

The paraffin material was cut at 6 to 12 μ thick. Both smears and sections were stained exclusively with Newton's iodine-gentian violet. Feulgen's reaction was also used.

DESCRIPTION OF MEIOSIS

Since there is no difference in the behaviour of the chromosomes in the different varieties, apart from that due to chromosome numbers, the following description is applicable to all except where otherwise stated.

First meiotic division.

Resting stage. At this stage the pollen mother-cells are polygonal and filled with dense protoplasm. The nucleus is more or less spherical and somewhat larger than that of the somatic cells. The cavity within the nuclear membrane is filled with a lightly staining material and a large deeply staining spherical nucleolus is situated in the centre (Pl. X, Fig. 1). It is very difficult to distinguish the chromosomes from one another. The reticulate appearance described by various workers at this stage was not seen in this material. No anastomoses were observed by Nebel (1932*a*) in the resting stage of Tradescantia, nor by Koshy (1934) in Allium.

Leptotene. With the approach of the prophase, the nucleus becomes larger and the submicroscopic threads of the resting stage become clearly visible as the delicate leptotene threads (Pl. X, Fig. 2). The threads are dispersed, without any orientation, throughout the nucleus. They can be followed to a considerable length, but their relationship to each other cannot be clearly made out. At this stage a deeply staining granule is visible attached to the nucleolus and connected with the chromatic thread (Pl. X, Fig. 2). This represents the nucleolar body first described by Latter (1926) in Lathyrus, and is called the nucleolar organizing body by McClintock (1934) in the satellited chromosomes of *Zea mays*.

Kaufmann (1931) in Tradescantia, Smith (1932) in Galtonia, Koshy (1934) in Allium, Hoare (1934) in Scilla, and Gates and Nandi (1935) in Oenothera describe the leptotene threads as double and presenting, like the early somatic prophase chromosomes, a beaded appearance due to the intertwining of the two chromonemata. In the present material no evidence could be obtained of their double nature. They appeared as single threads finely coiled. In fixed preparations these finely coiled threads have a beaded appearance, but when examined in iron-aceto-carmine, their spiral nature becomes clear (Pl. X, Fig. 3).[1] It seems therefore that the beaded appearance attributed by Kaufmann, Koshy, and others as due to the intertwining of the two threads, and by others as chromomeres, are presumably the optical images of the spiral twists of the threads which have been exaggerated by the action of the fixatives.

The synizetic contraction described by various investigators was not observed in well-fixed preparations in Hyacinthus, but there is no doubt a slight aggregation of the chromatic threads to one side of the nucleus. Taylor (1922) found that by adequate fixation the synizetic contraction was completely eliminated in Gasteria. Cleland (1924) regards synizesis as an artefact due to the influence of fixatives. Koshy (1934) in Allium and Gates and Nandi (1935) in Oenothera find no stage where the threads take the form of a knot. Huskins and Smith (1935) obtain no evidence of synizesis in Trillium. This is presumably a delicate stage of the nucleus when the threads become compacted together by the entrance of the fixative. In some nuclei the nucleolus by budding gives rise to a subsidiary nucleolus which soon assumes the same globular form as its parent nucleolus. Similar budding was observed by Gates and Latter (1927) in Lathyrus, Selim (1930) in *Oryza sativa*, Maeda (1930) in Sweet Pea, Koshy (1934) in Allium, Gates and Nandi (1935) in Oenothera, and Nandi (1936, unpublished) in rice.

Zygotene. The leptotene stage is of short duration, and soon it is seen that some of the threads are beginning to run parallel to one another along certain portions of their length, pairing closely with each other here and there (Pl. X, Fig. 4). This is the start of the zygotene stage. The homologous threads pair side by side. The mode of pairing is thus parasynaptic. The pairing begins at the ends of the chromosomes, as was observed by Gelei (1921) in Dendrocoelum, Belling (1931) in Liliaceous plants, Huskins and Hearne (1933) in Oats, Hedayetullah (1933) in *Oenothera missouriensis*, Koshy (1934) in Allium, and Nandi (1936, unpublished) in Oryza. Darlington (1935), from his observations in Fritillaria, believes that pairing begins at the spindle fibre attachment region. Huskins and Smith (1935), on the other hand, find indications of pairing beginning both near the spindle attachment and at the ends in *Trillium erectum*. In triploid Hyacinths three threads lie separately parallel to each other. They pair in such a way that only two lie together at any point and the third remains free (Pl. X, Fig. 5). Thus the genuine association is only between pairs. This is in agreement with Newton and Darlington's (1929) observation in triploid Tulips and Hyacinths.

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[1] In aceto-carmine preparations the nucleolus is usually invisible.

Pachytene. Soon the pairing of the threads becomes complete throughout their lengths. This is the pachytene stage (Pl. X, Fig. 6). The threads undergo thickening and shortening, presumably by the new coiling. Along their longitudinal axis the threads show a row of granules which no doubt represent the 'Chromatin Scheibe' of Strasburger (1888), the 'chromatic flakes' of Allen (1905), or the chromomeres of Belling (1928, 1931) and others. In well-fixed preparations the spiremes show a spiral structure in which the threads are coiled into a spiral of short pitch. The coiled structure becomes clearly visible in iron-aceto-carmine. Such spiral structure of the pachytene has been demonstrated by Babcock and Clausen (1929) in Crepis, Smith (1932) in Galtonia, and Shinke (1930, 1933) in Amaryllis and Sagittaria.

Huskins and Smith (1935) observe that before synapsis is complete in all regions the earliest synapsed chromomeres begin visibly to split, and so find no definite pachytene stage for the whole nucleus in Trillium. This is contrary to the observations in Hyacinthus, where all the homologous chromosomes are synapsed at the same time throughout the nucleus. Darlington (1935) finds that in all species of Fritillaria pairing is incomplete and is particularly liable to fail in the parts attached to the nucleolus. The transverse connexions observed by Belling (1933) between the so-called chromomeres at zygotene or pachytene were not seen in Hyacinthus.

Diplotene. The nuclei at this stage are usually collapsed, and consequently the internal structure of the chromosomes is obscured. Bělař (1928) has explained this condition as due to two processes going on side by side at the prophase of the division: one the reduction of viscosity in the nucleoplasm, and the other the increase in the viscosity of the chromosomes due to loss of water. He therefore states that a point is reached when the viscosity of the ground substance has been reduced without the chromosomes becoming rigid enough to stand the effect of the fixatives.

Pl. X, Fig. 7, represents the diplotene stage, where the spaces between the chromosomes have become evident and the threads fall apart from each other at certain places, forming loops. Each of the homologues now shows indications of doubleness. The bivalents thus show quadripartite structure, each homologue having divided into two chromatids. Each chromatid consists of a single spiral fitted into the spiral of its partner.

Diakinesis. The chromosomes reach their maximum lengthwise contraction due to the formation of the major coils. The internal structure of the chromosomes at this stage is usually obscured and the surface presents a corrugated appearance (Pl. X, Fig. 8). At late diakinesis the nucleolus disappears.

First metaphase. The chromosomes at the first metaphase, as in Tradescantia, are composed of major and minor spirals, as in the photomicrograph (Pl. XII, Fig. 32). They are called primary spirals or spirals of the higher order, and secondary spirals or spirals of the lower order, by Kuwada and Nakamura (1933). By ordinary methods of preparation the internal structure of the chromosomes at this stage is usually obscured. The turns of the spirals run so closely that they appear as a series of discontinuous bars, which in some favourable cases can be observed as a continuous spiral thread (Pl. X, Fig. 9; Pl. XII, Figs. 33, 34). Their coiled nature is revealed more clearly by pretreatment with ammonia vapour, which seems to dissolve the chromosome matrix and thus permit the spiral chromonemata to expand. Pl. XII, Figs. 33, 34, are photomicrographs of first metaphase chromosomes prepared by La Cour's (1935) method using ammonia vapour. Here the spirals appear to be singly coiled, but arrows indicate places showing minor spirals. The doubly coiled or 'spiral within spiral' nature of the chromosomes becomes clearly demonstrable by Kuwada and Nakamura's (1933) ammonia iron-aceto-carmine method (Pl. XII, Figs. 32, 35, 36, 37). The arrows indicate the minor coils. In Pl. XII, Figs. 38, 39, due to a longer exposure to ammonia, the major spirals in some chromosomes have become completely drawn out revealing the minor spirals only. In Hyacinthus the coiled chromosomes consist, as in Tradescantia and Lilium, of two chromatids coiled together so that it often appears as a single spiral thread. But in certain loci their double nature is recognizable (Pl. X, Figs. 9, 12; Pl. XII, Figs. 32, 33, 37). Darlington (1935) finds that in Fritillaria the paired chromatids are not coiled together but have separate spiral systems.

At this stage the chromosomes are composed of two substances, a less stainable matrix and a stainable chromatic spiral which winds around inside the matrix. Since the minor and major spirals maintain their shape without undergoing any considerable change, Kuwada and Nakamura (1934) suggest that these spirals have their own matrices in Tradescantia. The number of major coils in the larger chromosomes in Hyacinthus is about 10 per chromosome. The direction of coiling appears to be consistent on one side of the spindle attachment. Reversal in the direction of the coiling takes place in every chromosome at the spindle attachment only. At other loci there seems to be no change in the direction of coiling.

The metaphase configurations of chromosomes in diploid and triploid Hyacinths illustrated in Pl. X, Figs. 10, 11, resemble those described by Belling (1927, 1929), and Darlington (1929). One of the pollen mother-cells in the variety *Garibaldi* showed sixteen bivalents at metaphase, double that found in normal cells, and the cell itself was nearly double the size of the others (Pl. X, Fig. 12). This is probably a case of syndiploidy, where the two nuclei of the premeiotic division fused (fusion must have been subsequent to zygotene) to give double the number of chromosomes. This abnormality, which has been noted to occur in several plants, probably accounts for the formation of some polyploids. Sax and Humphrey (1935) observe that during metaphase in Tradescantia the coiled chromatids of each chromosome gradually separate until each homologue consists of two separated coiled threads. In Hyacinthus the chromatids remain coiled together throughout the metaphase stage.

First anaphase. As the chromosomes pass to the poles they become drawn out to a considerable extent. This drawing out is greatest in the proximal region (Pl. X, Fig. 13). When the tension is released the chromosome spirals come to their original form of uniformly larger gyres (Pl. X, Fig. 14; Pl. XII, Figs. 40, 41). This characteristic behaviour of the chromosomes at this stage is observed by Sax (1930) in *Lilium regale*, Taylor (1931) in Gasteria, Iwata (1935), and Kato and Iwata (1935) in Lilium. The two sister chromatids become separated from each other except at the spindle attachment (Pl. X, Fig. 13; Pl. XII, Fig. 40). Each homologue is therefore composed of two coiled chromatids. The chromosomes assume V- and J-shaped appearances according to the place of constriction. In anaphase also the chromosomes maintain their doubly coiled nature. In ordinary preparations they appear single-coiled, showing only the major spirals (Pl. XII, Figs. 40, 41). But when the major spirals are drawn out by the action of ammonia the minor spirals become visible. In Pl. XII, Fig. 42, some chromosomes are seen completely drawn out, showing the minor coils only, while other chromosomes show major spirals also. Huskins and Smith (1935) observe 'tertiary split' at this stage in Trillium. No such split was observed in Hyacinthus.

First telophase. When the chromosomes reach the poles they shorten in length and the coils of the spirals are brought close together (Pl. X, Fig. 15). Lenoire (1932) observed this shortening of the telophase chromosomes in the living pollen mother-cells of *Lilium candidum.* This stage probably corresponds to the *tassement polaire* of the somatic division. The coils are brought so close together that they may appear as discontinuous discs. A wall is laid down separating the two daughter-cells. Each chromosome group is surrounded by a membrane, which is hemispherical at the beginning but later becomes ovoid. The major coils expand and thus appear to unravel, and the threads assume irregular zigzag shapes. The minor coils then become clear (Pl. X, Fig. 16). Taylor (1922) considered the meiotic telophase chromosomes to be eight-partite. Nebel (1932) describes the division of the chromosomes as taking place at the telophase stage of both somatic and meiotic chromosomes, and so describes four independent spirals in each telophase chromosome in Tradescantia. No such telophase division of the chromosomes was observed in Hyacinthus.

Interkinesis. The nucleus now increases in volume and assumes an ovoid shape. The major spirals are further drawn out in loose coils and become fewer. The chromosomes present a knotted appearance; the knots no doubt represent the turns of the minor spirals (Pl. X, Fig. 17). Finally the threads become straightened and all trace of the major spirals is lost. It then becomes very difficult to trace the separate threads throughout their length as they come nearer and are superimposed upon one another, but their free ends can be seen here and there. In advanced interkinesis the minor spirals also unravel to a certain extent. The matrix seems to be lost and the threads become thinner. Nebel (1932*a*) describes the chromosomes at interkinesis as composed of four threads attached at the spindle fibre insertion. No anastomoses between the threads were observed at this stage.

Second prophase. When the nucleus enters the prophase it becomes larger and the spireme threads become conspicuous (Pl. X, Fig. 18). The threads undergo new spiralling, on account of which the relic spirals are thrown into coils of larger loops. At late prophase the chromosomes are much thicker and shorter than at early prophase.

Second metaphase. The chromosomes at this stage are thinner and longer than at the first metaphase. The chromonemata are singly coiled, the coiling corresponding to the minor coils of the first division (Pl. XI, Fig. 19; Pl. XII, Figs. 43, 44, 45, 46). The two chromonemata are attached to one another at the spindle attachment region only (Pl. XII, Fig. 43). This singleness of the spindle attachment region indicates that it is the last region to divide. The chromosome types are recognizable at this stage (Pl. XI, Fig. 19). Pl. XII, Fig. 46, represents the four types of chromosomes, the arrows indicating the constrictions. The number of minor spirals was ascertained to be about 34 in the long chromosomes, about 15 in the medium, and about 10 in the short chromosomes. The direction of coiling seems to be constant in each arm of the chromonema spiral thread, reversing its direction at the spindle attachment region only.

Second anaphase. At early anaphase the two half-chromatids are coiled together so intimately that they appear as a single coil (Pl. XII, Fig. 47). But at a later stage the evidence of their double nature becomes clear at certain places (Pl. XI, Fig. 20). The anaphase chromosomes at the second meiotic division have been described as bipartite in a number of plants. Taylor (1931) in Gasteria, Smith (1932) in Galtonia, Nebel (1932*a*) in Tradescantia, Koshy (1934) in Allium, Hoare (1934) in Scilla, Huskins and Smith (1935) in Trillium, and Sax (1935) in Rhoeo, have furnished ample evidence of their double nature. It is not possible to say with certainty when the split in the chromosomes occurred, although it may be suggested that, as in the somatic division, it might have first taken place at the prometaphase stage. Laggards are commonly observed at the second anaphase (Pl. XII, Fig. 47) and these lead to abnormal pollen formation. In many cases the laggards, which vary from a few to many, surround themselves by cell-walls and form microspores, with the result that a pollen mother-cell consists of more than the normal four spores. The lagging chromosomes are sometimes amputated by the interphase cell-wall (Pl. XII, Fig. 48), the arrow in the figure indicating the connecting portion.

Second telophase. As the chromosomes reach the poles they become contracted and each group is surrounded by a membrane. A wall is laid down in each daughter-cell dividing it into four microspores. In the early stages the nucleus is hemispherical; later it becomes spherical. The new nuclei then enlarge considerably. The chromosomes lengthen and the minor spirals uncoil, throwing the thread into a super spiral. The double nature of the thread is visible at early stages (Pl. XI, Fig. 21). But as the chromosomes lengthen the threads become closely approximated with each other and then they appear as single threads (Pl. XI; Fig. 22).

Supernumerary divisions.

In the variety *Nimrod*, specially prepared for early flowering, it was observed that soon after the meiotic divisions had been completed the four nuclei show evidences of another division. The chromosomes assume visible individuality and become thicker (Pl. XI, Fig. 23). They then arrange themselves at the equatorial plate (Pl. XI, Fig. 24). Pl. XI, Fig. 25, represents the telophase stage of the first supernumerary division, showing eight nuclei. The cells formed as a result of the first supernumerary division very soon go through a second division which is essentially similar to the first. Pl. XII, Figs. 49, 50, represent the telophase stage of the second division. Only two such divisions were noted. In some cases microspores of various sizes were observed within a single mother-cell. It is unknown whether this was the result of regular supernumerary division or of irregular meiotic divisions in consequence of the aneuploid nature of this variety. These divisions appear to be regular somatic divisions, where a definite division of the chromosomes takes place. This appears to be the first record of a regular polymitotic division. Beadle (1931) described supernumerary divisions in a polymitotic mutation of Maize. But he finds that the chromosomes are distributed at random to the two poles without splitting, as a result of which the number of chromosomes in the cells which are finally formed is much reduced, many of them containing a single chromosome. Beadle attributes these supernumerary divisions in Maize to a recessive gene.

Post-meiotic division. The prophase of the first division in normal pollen grains resembles the somatic prophase. Here also in the early prophase the two sister chromatids are so closely approximated that their double nature is not clear throughout the length of the threads, but at some places it may be seen (Pl. XI, Fig. 26). The relict and super spirals are more clearly visible.

Pl. XI, Fig. 27, represents a late prophase where the two sister chromatids have become more or less separated. In the prophase of the first division in the pollen grains the new coiling is clearer than in the prophase of the somatic division in root-tips. The structure of the chromosomes at metaphase and anaphase is usually obscured. This is presumably due to the fact that the pollen grains have a thick wall which prevents the fixatives from penetrating quickly.

In the variety *Captain Byoton*, in which $2n = 22$, the pollen grains showed chromosome numbers from 9 to 12, this being the result of irregular meiotic divisions (Pl. XI, Figs. 28–31). One of the chromosomes (Pl. XI, Fig. 30) showed two secondary constrictions.

DISCUSSION

Spiral structure of the chromosome.

It has already been pointed out that the leptotene threads are composed of finely coiled spirals. In fixed material these coiled threads show knotted appearances, which have been described as chromomeres by various investigators. Kaufmann (1931), Smith (1932), and Koshy (1934) regard them as due to the two chromatids intertwining closely. Chipman (1925) regarded the 'chromatic knots' in Lilium as simply spirally entangled portions of the chromatic threads. Szakien (1927) and Schaede (1928) interpreted them as optical illusions. Taylor (1931) in Gasteria, remarks that in exceptionally favourable instances the early leptotene structures which hold the stain are spiral. Shinke (1934) observes that in well-fixed preparations of Sagittaria the spireme shows spiral structures. Darlington (1935) in Fritillaria, and Huskins and Smith (1935) in Trillium, describe the leptotene threads as straight, showing no internal spirals but chromomeres. They express the opinion that the formation of the spirals coincides with the division of the chromosomes at pachytene. The same coincidence has been observed by them in the somatic division, where spiralling begins, according to Darlington, after the split of the chromosomes at the resting stage, and according to Huskins and Smith after the split at prometaphase. In the somatic study of Hyacinthus it was pointed out that with the commencement of the prophase each chromatid begins to undergo new spiralling. The same phenomenon takes place in the meiotic chromosomes. They begin to coil at the beginning of the prophase. To the author it appears that splitting and spiralling are two different phenomena induced by different forces. Spirality seems to be effected, as will be explained later, by the attraction of certain molecules in the chromosomes, whereas splitting appears to be the result of the duplication of the material composing the chromonema. If there is any relationship between the split and spirality, then a similar behaviour should be observed when the secondary coiling begins in the chromosomes of the first meiotic division.

A second coiling begins at diakinesis. Hence the first metaphase chromosomes are made of major and minor spirals. The chromosomes at this stage have been described as singly coiled by Maeda (1928) in Lathyrus, Taylor (1931) in Gasteria, Smith (1932) in Galtonia, Nebel (1932*a*) in Tradescantia, Hoare (1934) in Scilla, and Koshy (1934) in Allium. The spirals observed by these workers correspond to the major spirals of the author. With their ordinary methods of preparation they were unable to see the minor spiral. With the ammonia and other pretreatments it is now possible to observe, or find indications of, the minor coils.

According to Kuwada (1932), Fujii (1926) was the first to observe the first metaphase chromosomes of *Tradescantia reflexa* as doubly coiled. Ishii (1931) confirmed Fujii's observation in the same plant. Kuwada (1932) was able to demonstrate this 'double-coiled' structure very clearly by the ammonia method in *Tradescantia virginica*. Since then these double-coiled chromosomes have been seen by various Japanese and a few other workers in the same and other plants. They have been reported in *Tradescantia reflexa* by Kuwada and Nakamura (1933, 1934), Kato (1934), and Sax and Humphrey (1934); in *Sagittaria Aginashi* by Shinke (1934); in *Lilium longiflorum* by Shinke (1934), Kato and Iwata (1935), and Iwata (1935); in *Lilium tigrinum* by Shinke (1934), Kato and Iwata (1935), and Iwata (1935); in *Lilium speciosum* by Kato and Iwata (1935) and Iwata (1935); in *Hosta japonica* by Ishii (1931); in *Trillium kamtschaticum* by Matsuura (1935); in *Fritillaria* spp. by Darlington (1935), and in *Rhoeo discolor* by Sax (1935). Huskins and Smith (1935), in their study of the structure of the meiotic chromosomes in *Trillium erectum*, deny the existence of the minor coils. It appears that their 'tertiary split' is really the optical image of the minor coiling which becomes clear only with proper pretreatments. They also regard the minor spirals and their 'tertiary split' to be the 'alternative interpretations of the same optical images'.

No definite evidence could be obtained regarding the order in which the major and minor spirals develop. Kuwada and Nakamura (1933, 1934) state that the major spirals are formed primarily and the minor secondarily. They remark that at late prophase, when the matrix is abundant, the secondary coiling accompanied by its own matrix, develops. The primary coils are therefore prevented from being drawn out by the compressing force of the matrix. If this is so, then the number of coils in Kuwada's primarily formed spirals should be almost the same, or at any rate not less at metaphase than at prophase. The number of primary or major coils in the large chromosomes in Hyacinthus is about 10. This number of coils was determined in about ten chromosomes.

An exact count of the number of secondary or minor coils could not be made at metaphase I, but since the pitch of the minor coils at the first metaphase appears to be the same as that of the coils at second metaphase, it is suggested that the number is the same in both cases, that is thirty-four, which is the count for the large chromosomes at second metaphase, as determined by counts in six different chromosomes. This great difference in number suggests that the major coils, which Kuwada regards as primarily developed, are actually secondarily developed.

The chromosomes at the second metaphase and anaphase are slender and longer, and they have a larger number of coils than those of first metaphase. They are single-coiled like Tradescantia. The coiling corresponds to the minor coils of the first division. Tuan (1931), in an abnormal type of Gasteria, Shinke (1934) in Sagittaria, and Darlington (1935) in Fritillaria find no difference in the width of the spirals of the first and second metaphase chromosomes. They observe them also as doubly coiled. Kato and Iwata (1935) find two types of chromosomes at the second metaphase in *Lilium longiflorum* and *L. speciosum*. In some cases the chromosomes are slender and longer and are singly coiled, whereas in other cases they are thicker and shorter and are doubly coiled. In *L. tigrinum* they observe only double-coiled chromosomes.

The nature of the singly or doubly coiled second metaphase chromosomes probably depends upon the duration of the interphase. With a longer interphase the major coils are fully drawn out and so do not appear in the second division. With a shorter interphase both the coils appear.

A study of the number of coils at second metaphase in the three types of chromosomes (long, medium, and short) was made. It was found that the number of coils in a chromosome was a stable character and the pitch of the coils is almost the same in all chromosomes. The formula $L = n\sqrt{4(\pi r)^2 p^2}$ which was very kindly supplied by Professor G. F. J. Temple, King's College, was applied to calculate the total length of the chromonema thread at second metaphase. L in the formula denotes the total length of the chromonema, n = number of coils, p = pitch, or the distance between the gyres, and r the radius. By measuring the actual length of the chromosome, the shortening of the chromosome at the second division due to coiling is calculated. Matsuura (1934) finds the ratio between the total length of the coils and the length of the chromosome at first metaphase to be 7·7 : 0·79 in *Trillium Kamtschaticum*. Table I indicates the relative lengths and contraction of the chromonema threads. It will be seen that the chromonema is about 6·5 to 7·3 times the length of the chromosome.

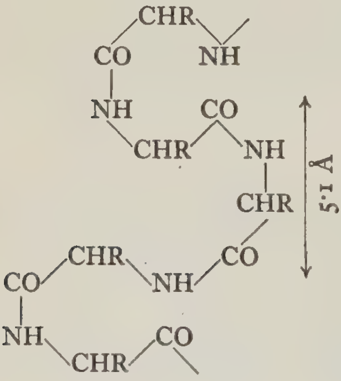
Contraction of the meiotic chromosomes.

During the prophase stages of meiosis we find that there is a gradual contraction of the chromonema thread, which reaches its maximum contraction at the first metaphase. Belling (1928) believed that the approximation of the chromomeres decreases the total length of the pachyphase thread to one-third of its original length in the meiotic chromosomes of Lilium, and that the rest of the contraction, which gives a chromosome approximately one-tenth of its original length, is effected by the chromonema coiling. Bridges (Alexander, 1928), on the other hand, assumed that the gene thread maintains approximately the same length at all stages in the chromosome cycle and that coiling is the primary factor in the contraction of the chromosomes. Sax (1935) believes that three factors are involved in the contraction of meiotic chromosomes in *Rhoeo discolor*—firstly linear contraction of the gene string, secondly the major, and thirdly the minor coiling of the chromonema thread. The author is of the opinion that the gene thread maintains the same length throughout the chromosome cycle, as was assumed by Bridges, and the factors responsible for the extreme contraction of the chromosomes at the first metaphase in Hyacinthus are the development of major and minor coils. This view is substantiated by recent researches in protein analysis. Animal hairs, nails, horns, spines, &c., are fundamentally the protein α-keratin. β-keratin, an extended form, is obtained by stretching α-keratin by steaming. The following diagram is a suggestion taken from Astbury (1931) of the intramolecular transformation in stretching.

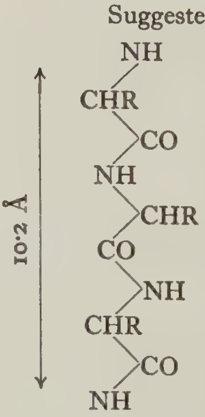
TABLE I

Showing the Ratio between the Relative Length of the Chromonema Thread and the Contracted Chromosome at Second Division

Types of chromosome.	Number of coils per chromosome.	Radius, ×2,100.	Pitch or distance between the gyres. ×2,100.	Total calculated length of the chromonema spiral. ×2,100.	Measured length of the chromosome. ×2,100.	Ratio between contracted chromosome and chromonema thread.
Long . .	34	1·5 mm.	1·5 mm.	324·36 mm.	50 mm.	1 : 6·49
Medium .	15	1·5 mm.	1·5 mm.	143·1 mm.	22 mm.	1 : 6·50
Short . .	10	1·5 mm.	1·5 mm.	95·4 mm.	13 mm.	1 : 7·3



α-Keratin



β-Keratin

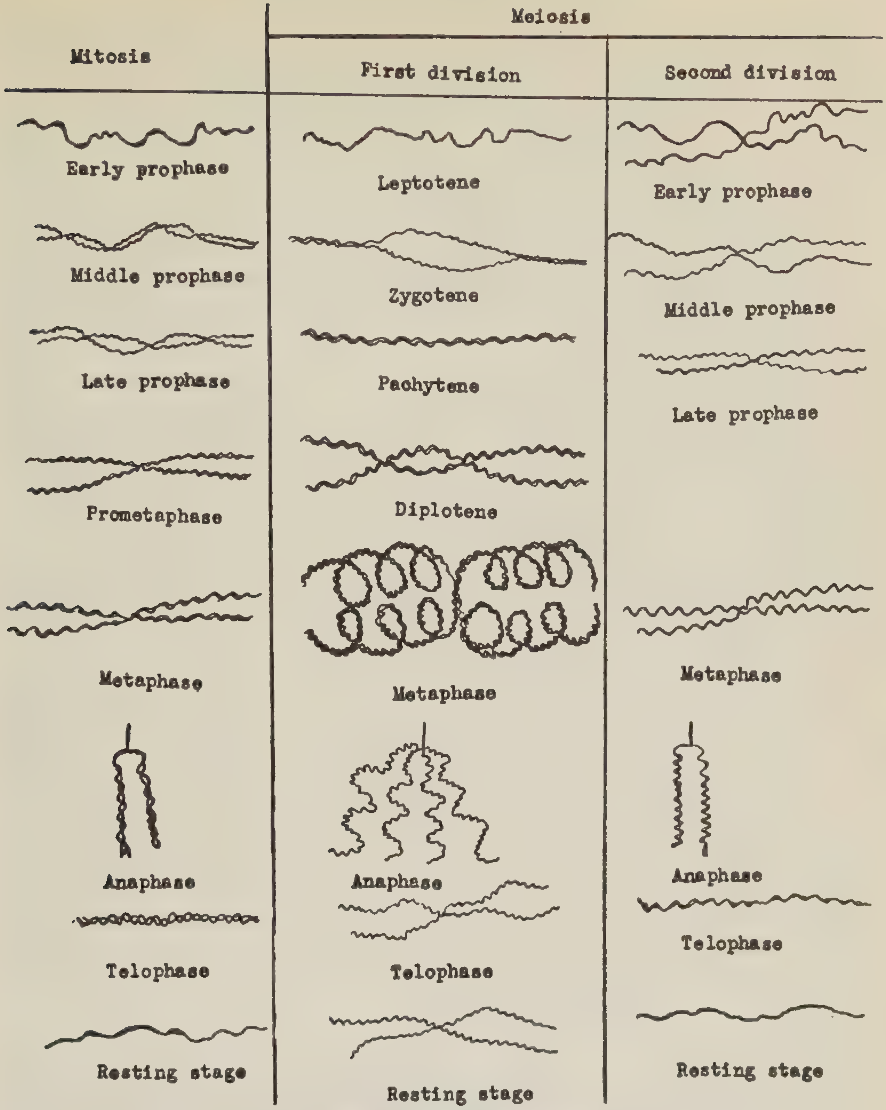


Diagram to show the history of spiral structures in the nuclear cycle in mitosis and meiosis.

Mechanism of spiralization.

So far no satisfactory explanation has been put forward regarding the cause of the coiling of chromosomes. Sax and Humphrey (1934) state that coiling may be induced by compressing the closely associated chromatids within the limiting pellicle in such a way that the chromatids are not permitted to rotate. Huskins and Smith (1935) assume 'that growth in thickness of the half-chromatids occurs during metaphase and anaphase on the outer surface of the chromatids'. They then explain that 'the extra-chromatin newly laid down on each half-chromatid will sooner or later be expected to undergo syneresis and this will provide a very self-perpetuating mechanism for spiralization'. These mechanical interpretations do not explain why coiling takes place. Kuwada (1935) explains the coiling to be due to an internal factor. Darlington (1935) concludes that spiralization is associated with a compensating internal twist or molecular spiral due to a rearrangement of the constituent particles, a rearrangement either between molecules or within molecules. The molecular spiral itself he assumes to be conditioned by change in the surface charge or double electric layer on the chromosomes, which change he supposes to be taking place during prophase. Wrinch (1935) has suggested various contractile factors which she believes the chromosome micelle may be expected to possess. These intramolecular or intermolecular factors depend upon the salt-forming attractions between basic and acidic ionized groups; the variation in the number of ionized groups belonging to single molecules, which number varies with pH and also with the type of linkage in the molecule. It may be suggested here that change in physico-chemical conditions involving attraction and repulsion between certain molecules situated at regular intervals along the chromatids could account for both the close spiralization and the straightening out of the threads.

Direction of coiling. The direction of coiling of the major spirals usually appears to be in opposite directions in the paired homologous chromosomes of a bivalent in Hyacinthus. Sufficient observations were not available to make a definite statement on this question. Huskins and Smith (1935) observe the paired meiotic chromosomes of *Trillium erectum* very frequently coiling in opposite directions. Nebel (1932*b*) and Sax and Humphrey (1934) in Tradescantia, and Sax (1935) in *Rhoeo discolor*, find the direction to be at random for the two homologous chromosomes of each bivalent. Matsuura (1935) in *Trillium Kamtschaticum* observed no definite relation in the direction of coiling in the two homologues.

The direction of coiling within a single meiotic chromosome on either side of the spindle attachment appears to be constant in Hyacinthus. The change in direction takes place at the spindle attachment only. Nebel (1932*b*) found in Tradescantia 83 chromosomes with the direction of coiling unchanged in the two arms of the chromosomes and 61 with a reversal of coiling at the fibre attachment. Sax and Humphrey (1934), in their analysis of 128 chromosomes in Tradescantia, observe the direction of coiling to be the same in 90 chromosomes and reversed at the fibre attachment in 38 chromosomes. Sax (1935) finds that the direction of coiling may change at the spindle attachment in *Rhoeo discolor* but is seldom reversed at other loci. Huskins and Smith (1935) find frequent changes in the direction of coiling in the anaphase chromosomes of *Trillium erectum*, the changes being associated with chiasmata. Matsuura (1935) also finds that in *Trillium Kamtschaticum* the direction of coiling may change at any point, but he observes a certain restriction in the occurrence of the reversal, in such a way that a certain number of coils must intervene between one reversal and the next. From his observations he comes to the conclusion that the direction of coiling is not a stable character for a given chromosome.

SUMMARY

From a detailed cytological investigation of the meiotic chromosomes of Hyacinthus various conclusions have been reached.

The leptotene threads appear to be single and show a finely coiled (minor) spiral structure.

The pairing at zygotene is parasynaptic, pairing beginning from the ends of the chromosome threads.

The chromosomes at diplotene are quadripartite, the division in the threads taking place just prior to this stage. The major coiling develops at diakinesis.

The first metaphase chromosomes are doubly coiled, the major with a smaller number and minor with a larger number of coils. The two chromatids in each homologue are coiled together (i.e. parallel to each other) in the chromonema throughout metaphase. They become separated at first anaphase, remaining attached to one another only at the spindle attachment region.

The major coils are drawn out at the proximal end in early anaphase. At late anaphase the coils again become regular. The minor spirals both at first metaphase and anaphase become observable by ammonia treatment.

The major coils are drawn out at first telophase revealing the minor spirals, which also become unravelled to a certain extent in the interkinesis.

At second meiotic division new spirals are formed which uncoil the relict spirals. These spirals are smaller and more numerous than the major spirals of the first division. The second metaphase and anaphase chromosomes are therefore composed of minor spirals only.

The second anaphase chromosomes are double, but the two chromatids are coiled together so closely that they are not easily observed. Laggards producing abnormal structures are common.

In the flowers from the specially treated bulbs of the variety *Nimrod* the microspore quartet were observed to undergo two supernumerary mitoses involving a regular split in all the chromosomes.

In the post-meiotic divisions the chromosomes are similar in structure to those of a somatic division.

The chromomeres are shown to be an optical effect produced by the finely coiled spirals.

The extreme contraction of the chromosomes at first metaphase is attributed to the development of the major and minor spirals.

The direction of coiling appears to be constant on the two arms of the chromosomes, reversal taking place at the spindle attachment only.

The chromonema is found to be 6·5 to 7·3 times the length of the chromosome at metaphase II.

It is suggested that attraction and repulsion between protein molecules situated at regular intervals along the chromatids could account for both the close spiralization and elongation of the chromonema thread.

In conclusion I wish to express my heart-felt thanks to Professor R. Ruggles Gates, for his criticisms and helpful suggestions throughout the course of these investigations. I also wish to acknowledge my indebtedness to the University of Allahabad and the Chandra Bellabh Memorial Trust, Garhwal, for affording me facilities to undertake this work in King's College, University of London.

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EXPLANATION OF PLATES X TO XII

Illustrating Dr. S. P. Naithani's paper on 'Chromosome Studies in *Hyacinthus orientalis* L. II. The Meiotic Chromosomes.'

All figures were drawn with the aid of Reichert camera lucida at table level, using a Busch $\frac{1}{12}$-inch oil immersion, N.A. 1·30, Zeiss compensating oculars K. 12 (15×) or K. 20× and immersed Beck's condenser N.A. 1·40.

Figs. 9, 13, and 14 ×2,870.

Other figures ×2,100.

PLATE X

Fig. 1. Pollen mother-cell at resting stage.

Fig. 2. Leptotene stage, note free ends and nucleolar body.

Fig. 3. Leptotene stage in iron-aceto-carmine.

Fig. 4. Zygotene, pairing taking place in the diploid, var. *Garibaldi*.

Fig. 5. Zygotene pairing in triploid (*Queen of the Pinks*).

Fig. 6. Early pachytene, pairing complete in all the chromosomes (diploid).

Fig. 7. Diplotene, loops opening out between the pairs of chromosomes.

Fig. 8. Diakinesis, internal structure obscured, chromosomes contracted.

Fig. 9. First metaphase, treated with NH_3 vapour, fixed in 2 BE and stained in iodine-gentian violet, major spirals visible.

Fig. 10. First metaphase, eight bivalents in *Garibaldi* (diploid).

Fig. 11. First metaphase, eight trivalents in *Queen of the Pinks* (triploid).

Fig. 12. First metaphase, in giant cell with sixteen bivalents in *Garibaldi* (syndiploidy).

Fig. 13. Chromosome at first anaphase, early stage. Major spirals are drawn out in the proximal region, the two chromatids have separated from each other except at the spindle attachment region.

Fig. 14. Chromosome at first anaphase, later stage, the coils have taken the regular spiral form.

Fig. 15. Early telophase, showing polar clumping, eight chromosomes in each group.

Fig. 16. First telophase, the major spirals gradually uncoiling and the minor spirals becoming visible.

Fig. 17. Interkinesis, major spirals are completely drawn out and the minor spirals are more clearly visible.

Fig. 18. Second prophase showing eight chromosomes. The relict spirals are thrown into coils of larger loops due to the development of the new coiling, the two chromatids are attached with one another at the spindle attachment region only.

PLATE XI

Fig. 19. Three types of chromosomes at second metaphase showing reversal in the direction of coiling at the spindle attachment region only, and singly coiled minor spirals.

Fig. 20. Second anaphase, double nature of the chromosomes evident at some places.

Fig. 21. Second telophase, early stage, from *Queen of the Pinks* ($3n$). The minor spirals are thrown into spirals of larger loops, dual nature of the chromosomes evident.

Fig. 22. Second telophase in diploid, late stage, further drawing out of the minor spirals, duality indistinct.

Figs. 23–5. Stages of supernumerary divisions in the variety *Nimrod*. Fig. 23. Prophase of the first supernumerary division. Fig. 24. Metaphase of the first supernumerary division. Fig. 25. Telophase of the first supernumerary division.

Figs. 26 and 27. Successive stages of prophase in the pollen-grain mitosis (first division after meiosis) in *Yellow Hammer*. Fig. 26. Early prophase, relict spirals more distinct, in *Captain Byoton*. Fig. 27. Late prophase, relict spirals have disappeared, and the new fine coiling is appearing.

Figs. 28–31. Metaphase, in pollen grains of the variety *Captain Byoton*. Fig. 28. Has nine chromosomes. Fig. 29. Has ten chromosomes. Fig. 30. Has eleven chromosomes. Fig. 31. Has twelve chromosomes.

PLATE XII

All figures are photomicrographs taken with a Busch $\frac{1}{12}$-inch oil immersion, N.A. 1.30, and Zeiss compensating ocular 6. The photomicrographs were taken by Mr. C. S. Semmens, to whom I am much indebted for the skill and care exercised.

Figs. 32–7. First metaphase chromosomes, showing double-coiled structure, arrows indicate the minor coiling. Figs. 32, 35, and 37 are treated with ammonia vapour and stained in iron-aceto-carmine. × 2,000. Figs. 33 and 34. Treated with ammonia vapour, fixed in 2 BE and stained with iodine-gentian violet. × 2,800. Fig. 36. Treated with ammonia vapour and stained with iron-aceto-carmine. × 2,300.

Figs. 38 and 39. First metaphase chromosomes, treated with ammonia vapour and stained in iron-aceto-carmine. Due to long exposure to ammonia, the major spirals in some chromosomes are completely drawn out, revealing the minor spirals only. × 1,150.

Figs. 40 and 41. First anaphase chromosomes showing major spirals. Treated with ammonia vapour, fixed in medium Flemming and stained with iodine-gentian violet. × 2,800.

Fig. 42. The same. Due to longer exposure in ammonia vapour, the major spirals in some chromosomes are completely drawn out. × 1,150.

Fig. 43. Second metaphase exposed to ammonia vapour and stained in iron-aceto-carmine. The chromatids are attached to each other at the spindle attachment region only. × 1,150.

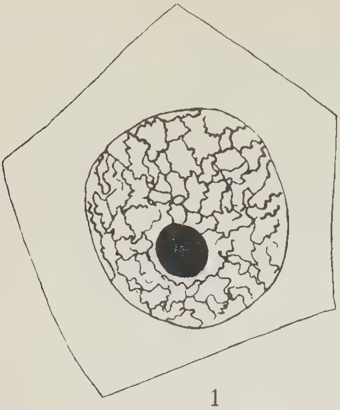
Figs. 44 and 45. Second metaphase chromosomes, aceto-carmine preparation, late stage showing slender chromosomes, single coiled (minor coiling). × 2,200.

Fig. 46. Iron-aceto-carmine preparation showing the four types of chromosomes, reversal in the direction of coiling at the spindle attachment region. × 2,300. (Cf. Pl. XI, Fig. 19.)

Fig. 47. Iron-aceto-carmine preparation, second anaphase, some chromosomes lagging. × 1,150.

Fig. 48. Second metaphase with one chromosome cut in two by the cell wall. × 1,150.

Figs. 49 and 50. Telophase of the second supernumerary division in the variety *Nimrod* in two foci, showing sixteen cells. × 920.



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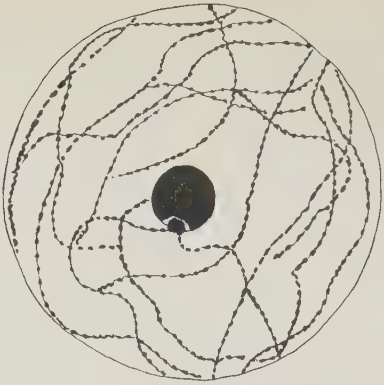

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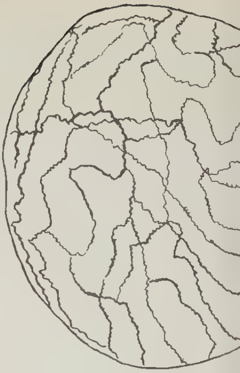

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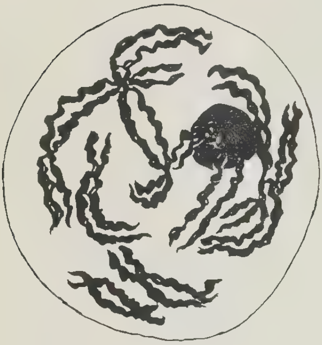

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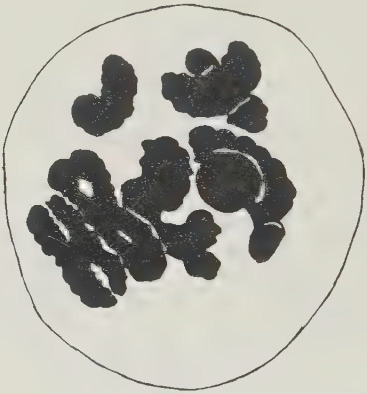

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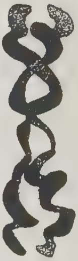

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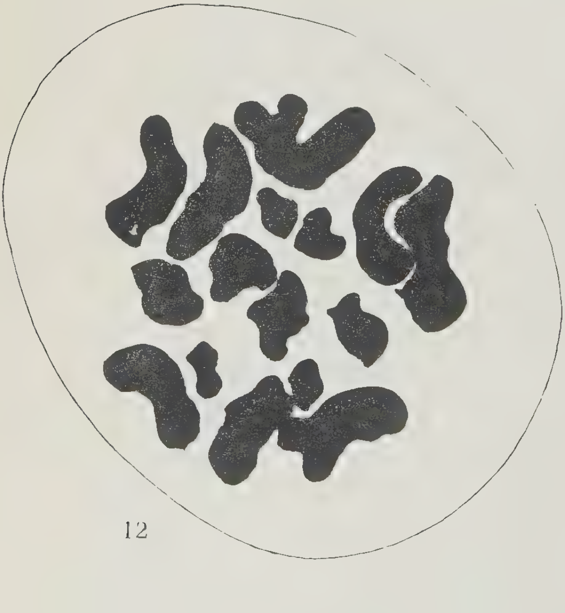

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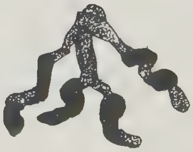

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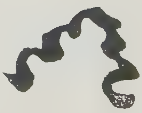

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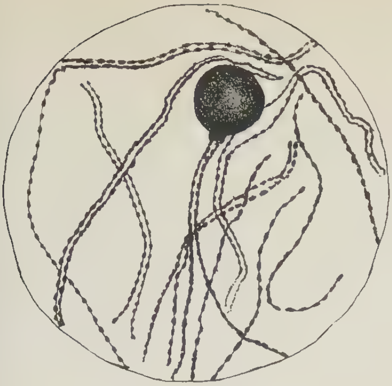
NAITHANI — MEIOSIS IN HYACINTHUS.



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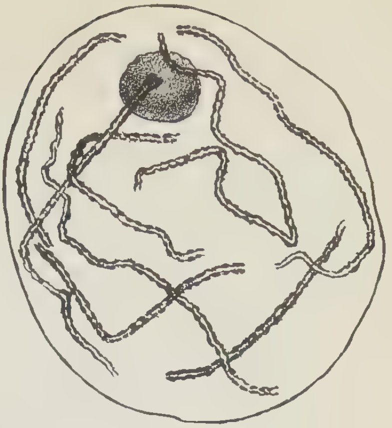

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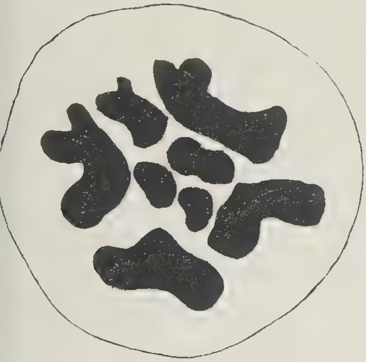

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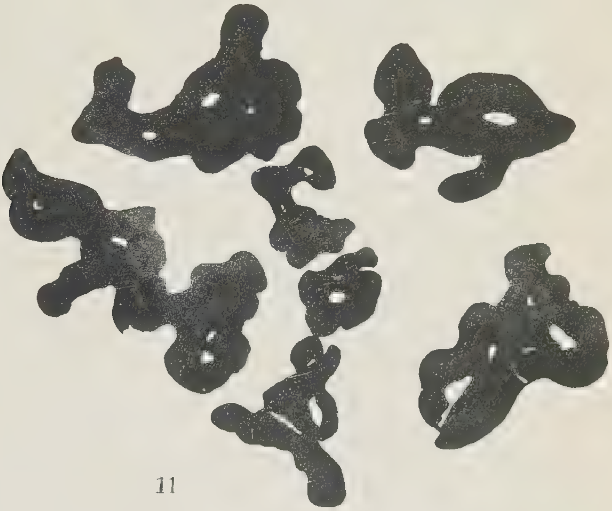

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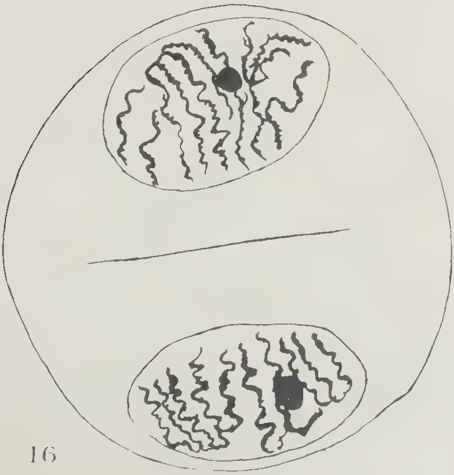

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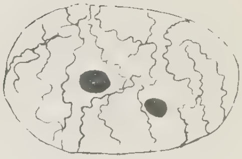

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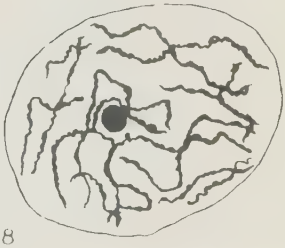

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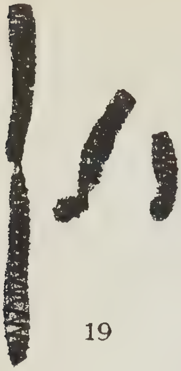

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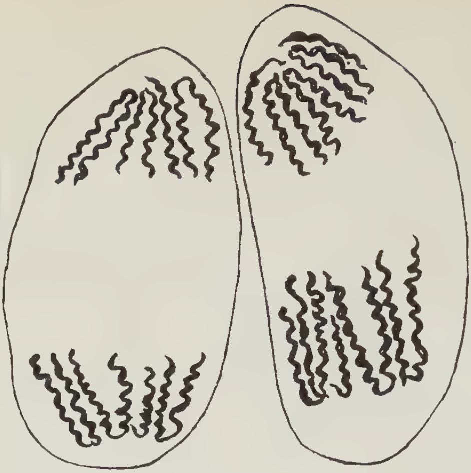

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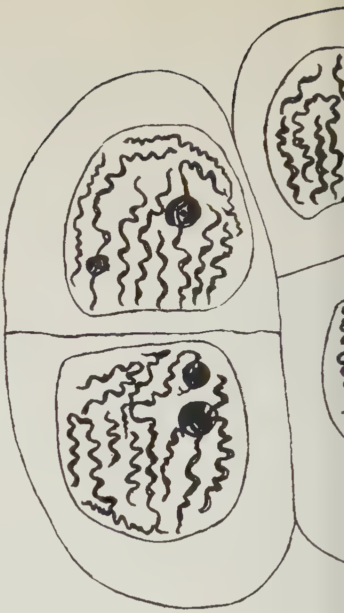

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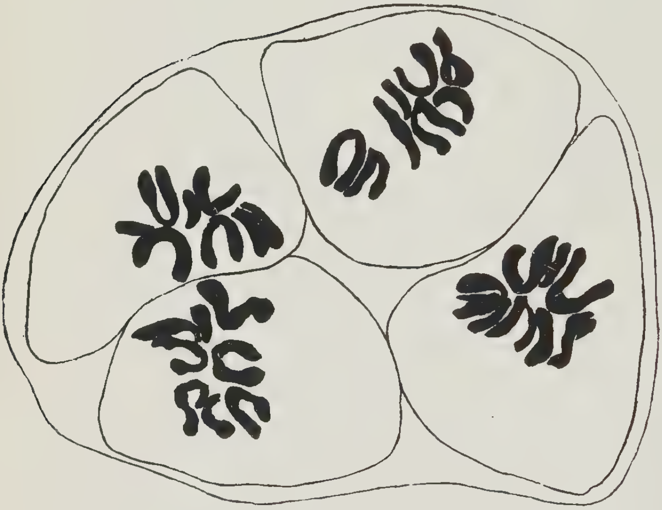

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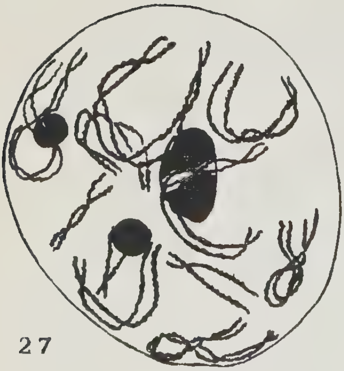

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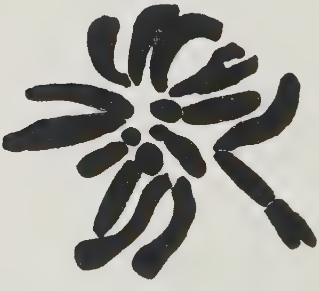

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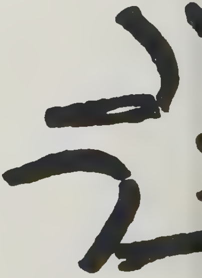

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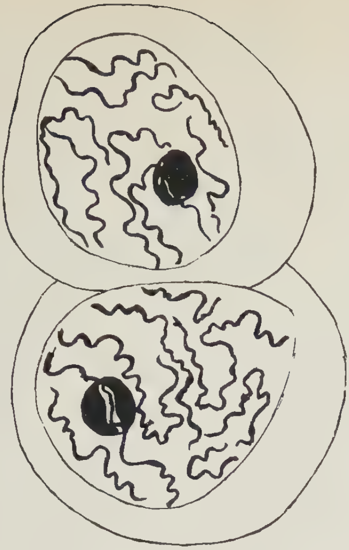
NAITHANI — MEIOSIS IN HYACINTHUS.



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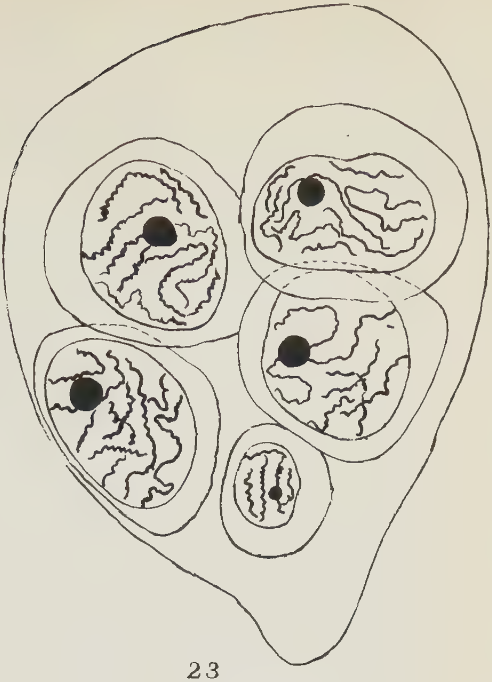

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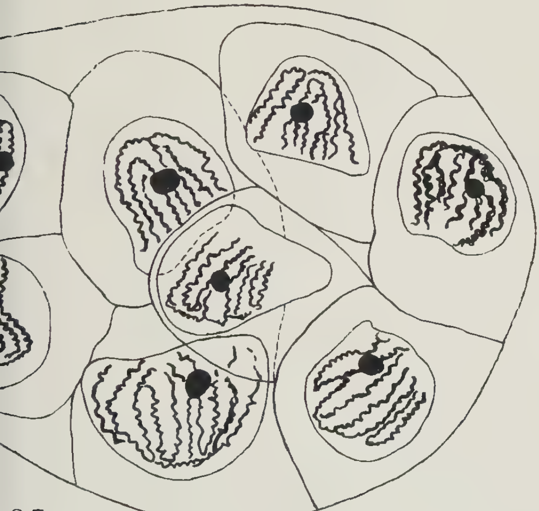

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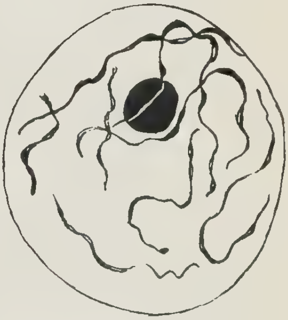

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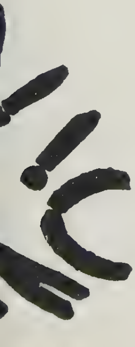

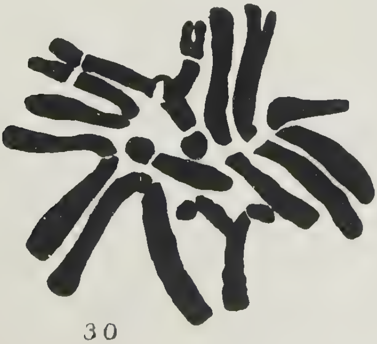

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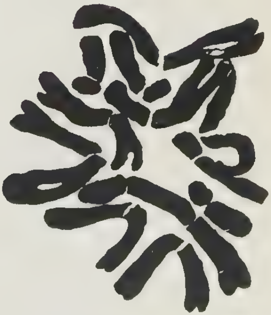

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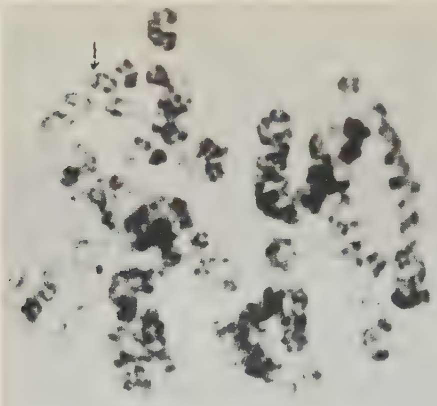

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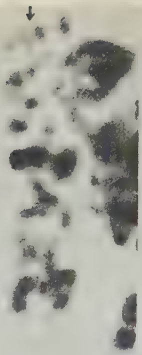

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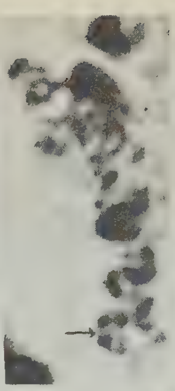

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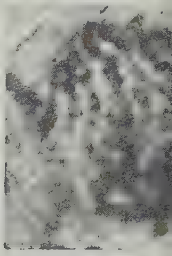

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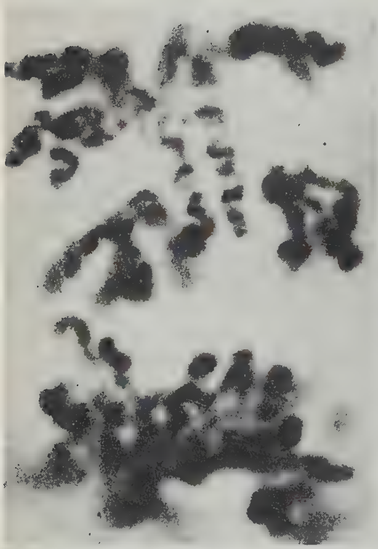

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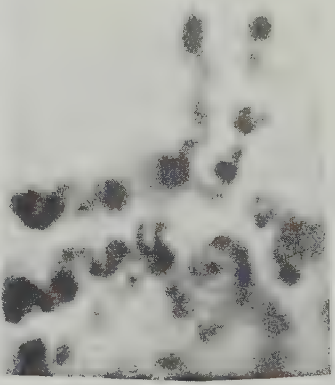

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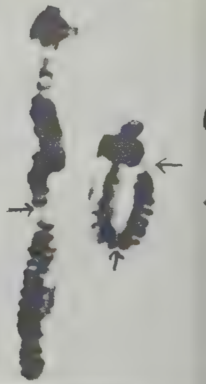

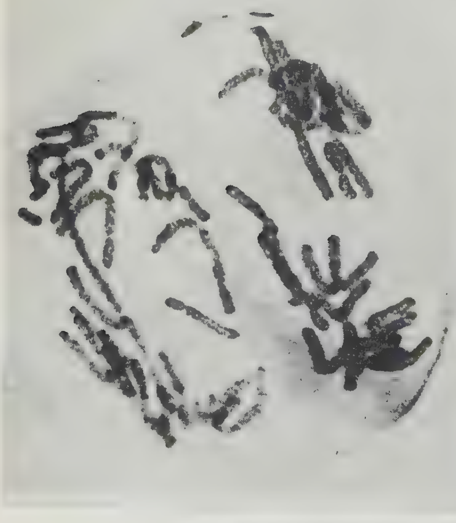

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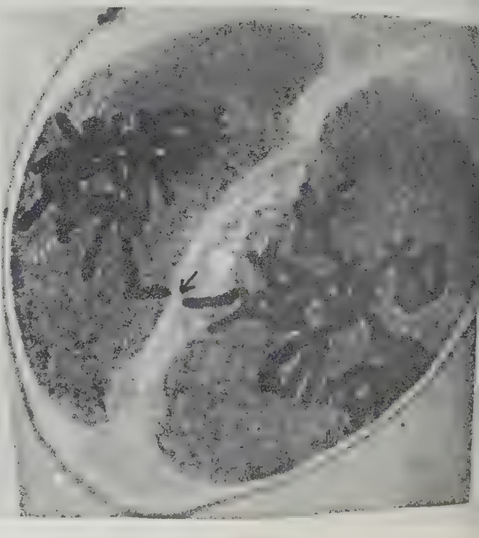

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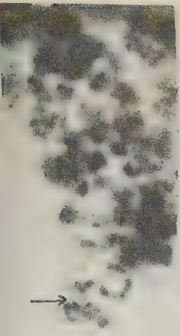
NAITHANI — MEIOSIS IN HYACINTHUS.



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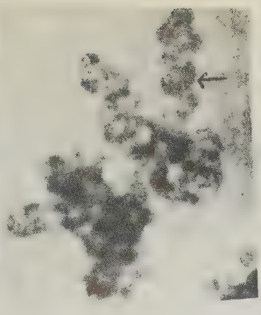

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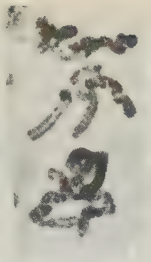

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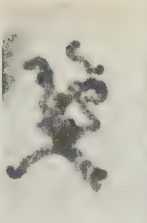

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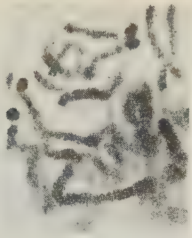

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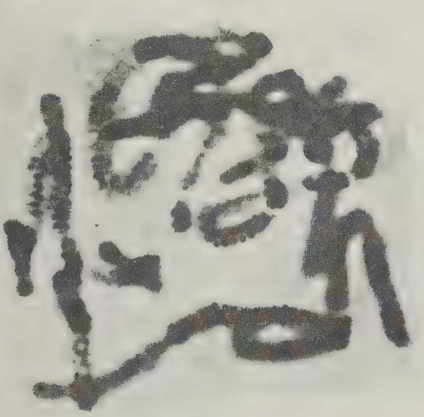

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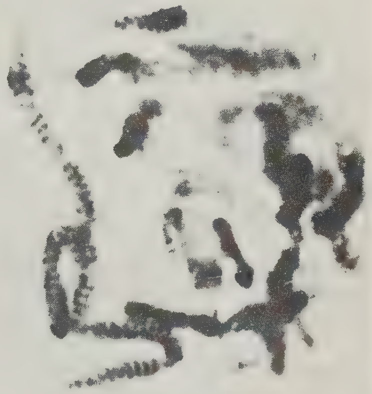

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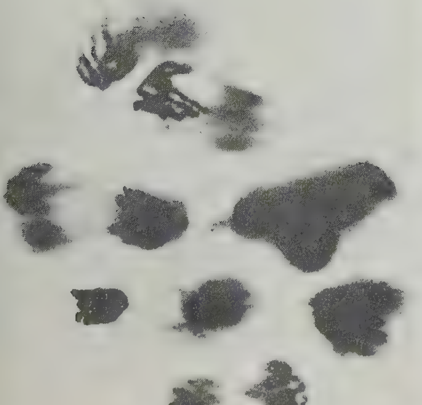

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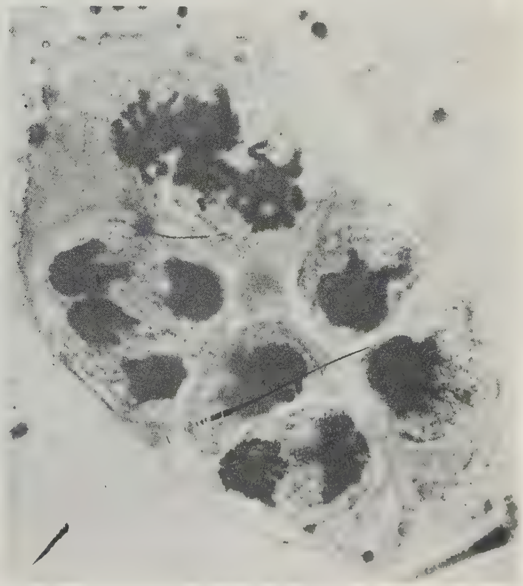

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Variations in the Leaves of Cotton Plants Grown under Irrigation in the Sudan Gezira

BY

G. B. PORTSMOUTH

(*Agricultural Research Service, Sudan*)

With nine Figures in the Text

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I. INTRODUCTION

THE leaves of the higher plants provide, in some form or another, what is perhaps the most frequently used source of material in physiological research. In all the vast accumulation of literature that has arisen it is far too frequently assumed that any two or more leaves of similar shape and appearance will be similar in their physiological characteristics. Age is perhaps the one factor that is generally recognized as giving rise to physiological differences between leaves, but in much of the work dealing with the influence of leaf age the problem is simplified by taking advantage of the fact that the leaves present on a shoot at any one time constitute an age series in themselves. Underlying all work of this nature, however, is the assumption that each leaf on the plant begins its history with the same constitution and repeats, in its turn, the same series of physiological changes at the same rate as every other. Richards (1934), in a detailed discussion of the subject, has pointed out that this assumption is entirely unwarranted, and has shown in a number of papers dealing with barley (Gregory and Richards, 1929, and Richards, 1932 and 1936) that successive leaves are so unlike in almost all

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the characters studied as to invalidate any attempt to deduce the age sequence of a single leaf from the particular values observed in successive members at any one time.

In the case of cotton, mention may be made of a paper by Eaton (1930) in which measurements of cell-sap concentration and leaf temperature are presented for successive groups of leaves along the main axis. The leaves measured varied in age from three to eighty-two days, and although full details of the nodal position are given for each group, he apparently regards them as successive steps in a simple age series. Hawkins (1927) used the topmost mature leaves to compare two varieties of cotton. Taking samples over a period of twenty-four days, he found that the average amount of water in the leaves of Acala cotton was always greater than in those of Pima. The value of this result is, however, somewhat minimized by his subsequent statement that the average Acala plant had 170 leaves as compared with only 103 in the Pima, since in these circumstances it would be surprising to find no marked differences in the fundamental characteristics of the leaves sampled.

These two examples will suffice since Richards, in the paper originally mentioned, has fully analysed a number of similar examples from the literature. The present paper details the results of a series of observations on area, dry weight, water, and nitrogen content of cotton leaves undertaken with the object of showing that in the cotton plant not only do the successive leaves differ from their inception in their individual characteristics, but that the course of the subsequent changes passed through by each individual leaf also differs for different leaves.

II. Experimental Methods

Observations were carried out during the two seasons 1934–5 and 1935–6 on cotton plants grown under irrigation at the Gezira Research Farm, Wad Medani. The variety employed was X 1530, a new Sakellaridis type selected at this station by A. R. Lambert. The main sowings in both seasons were in mid-August, which is approximately the optimum date for this area, while in the first season a second sowing was made a month later in mid-September.

It may be explained that in the Gezira cotton is normally sown during the rains in August, and that irrigation begins as soon as the cessation of the rains renders it necessary; this is usually towards the end of September. The main period of vegetative growth of the crop generally lasts for about three months until the onset of maturation coupled with the cooler climatic conditions brings it to a standstill. A further period of secondary growth usually follows in the new year after the opening of the first crop of bolls.

Experimental work was divided into two sections. For leaf-area determinations five normal plants were selected at random in the field and brought into the laboratory where the leaves were removed and grouped according to their nodal position. Areas were recorded by the sunprint method, after which the leaves were dried at 100° C. and weighed. For observations on water content the selected leaves were cut off in the field and rapidly transferred to airtight glass weighing bottles or tins, according to size. Each sample was divided into two replicates of five leaves each, which were taken from the same nodal position on ten random plants. The samples were weighed as quickly as possible after separation from the plant, the moisture driven off at 100° C., and the leaves finally re-weighed. All dried material was subsequently ground up and sent for nitrogen analysis to the Chemical Analytical Section of this Service at Khartoum.

III. MORPHOLOGICAL CHANGES

(a) *General description of the cotton plant.*

Cotton normally has a single main stem whose growth is continued indefinitely throughout the life of the plant. The number of nodes laid down by the terminal meristem during the main growing period naturally varies between rather wide limits, but 30–33 will represent the approximate range covered by the variety under consideration. One leaf is borne at each of these nodes, so that a continuous succession of new leaves is produced throughout the season. The buds in the axils of these leaves eventually develop into sympodial fruiting branches, but production of these branches does not usually occur below the 10th–12th node. Each fruiting branch may have any number of nodes, from two to six or even more, and, besides a flower bud, produces a leaf at every one of them. These leaves, however, are considerably smaller than those on the main axis, and during the main growing period make up only a small portion of the total leaf surface of the plant.

The leaves of the main axis do not persist indefinitely, and as new leaves are produced the earliest formed tend to be shed, so there is a continually increasing number of blank nodes at the base of the plant. The number of main axis leaves present at any one time, therefore, tends to become more or less constant. In the present investigation the plants were seldom found to be carrying more than fifteen functional leaves on the main stem.

(b) *Form of the main axis leaf.*

In order to compare the shapes of the different leaves it will be convenient to start at the lowest node and work up the stem towards the apex. If this course is adopted, it will be observed that the form of the successive leaves becomes progressively more and more complex. Leaf number[1] 1 and the two or three following it are simple, entire, cordate leaves. At about leaf number 5 the first indication of the characteristic lobing appears in the shape of two small lateral points. These rapidly increase in size as successive leaves are produced, so that the full trilobed form is reached with leaf 7 or 8. The shape now continues unchanged for several leaves when two more lateral points make their appearance at about leaf 15. These again rapidly develop into lobes, and from about leaf 20 onwards the five-lobed form continues unchanged.

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[1] All leaves are numbered from the basal node upwards, so that the oldest leaves have the lowest leaf number.

(c) *Maximal size of leaves. Total leaf area.*

The ultimate size reached by any leaf appears to depend primarily on its position with respect to the plant as a whole. In the mature plant the largest leaves are borne on the middle region of the stem and a rapid falling off in individual area occurs as either the basal or terminal regions are approached. This relation is well brought out in Fig. 1, which shows the maximal area recorded for each leaf during the whole period of observation in the season 1935–6.

TABLE I

Areas (sq. cm.) of Individual Leaves. (Average of 5 plants)

Date recorded.	Leaf No. 5.	Leaf No. 10.	Leaf No. 15.	Leaf No. 20.	Leaf No. 25.
25.9.35	57·8	(17·3)	—	—	—
2.10.35	60·6	(86·3)	—	—	—
9.10.35	*63·9*	*143·0*	(70·9)	—	—
16.10.35	60·3	130·4	(112·0)	(6·3)	—
23·10·35	Shed	130·4	152·8	(47·2)	—
30.10.35	—	90·4	146·2	(81·4)	—
6.11.35	—	135·0	*171·0*	106·6	—
13.11.35	—	137·0	156·6	113·8	(7·6)
20.11.35	—	135·6	152·4	99·8	(14·5)
27.11.35	—	Shed	167·2	114·6	66·0
4.12.35	—	—	145·2	*135·4*	63·3
11.12.35	—	—	169·4	121·4	*75·7*
18.12.35	—	—	133·0	103·4	58·2
25.12.35	—	—	—	—	—
1.1.36	—	—	140·4	106·0	51·2
Mean area*	60·7	128·8	152·4	112·6	62·9

* The mean area does not include areas of the immature leaves enclosed in brackets.

As measurements had to be made on a fresh series of plants each time, there was naturally some variation in the areas recorded. The extent of this variation can be gauged from Table I, which gives the actual figures obtained for a few selected leaves. Maximal areas are italicized.

As the season advances the total area of the leaves on the lateral branches continues to increase while, as previously stated, both the number and total area of the main axis leaves becomes almost constant. The amounts contributed by these two classes of leaves to the total leaf surface of the plant are shown graphically in Fig. 2. The data are those of season 1935–6. The figure also shows the extent of the main axis leaf shedding. The proportion of this occurring will, of course, vary considerably from season to season, since it is to a large extent the result of insect and disease damage, but the amount illustrated may be considered typical of the locality.

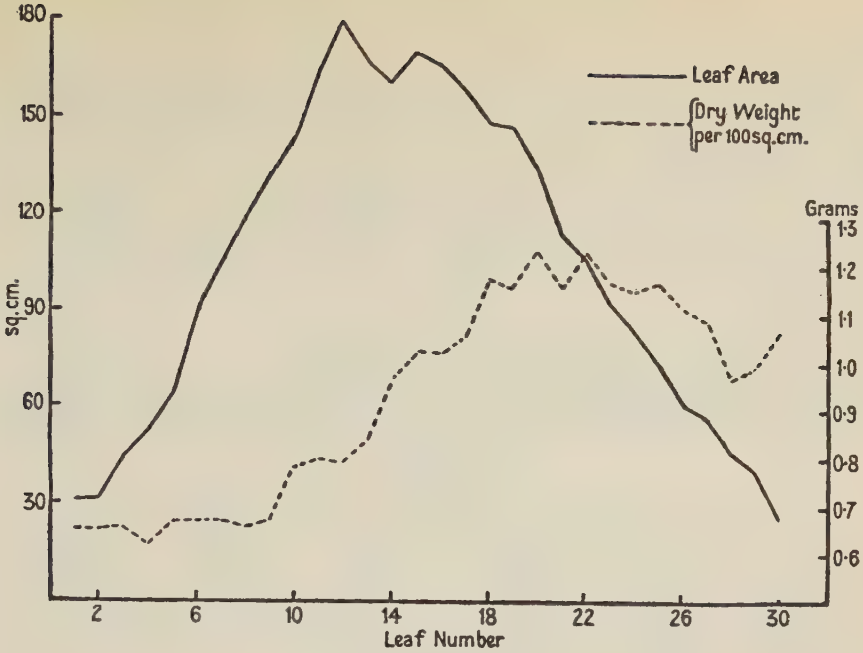

FIG. 1.

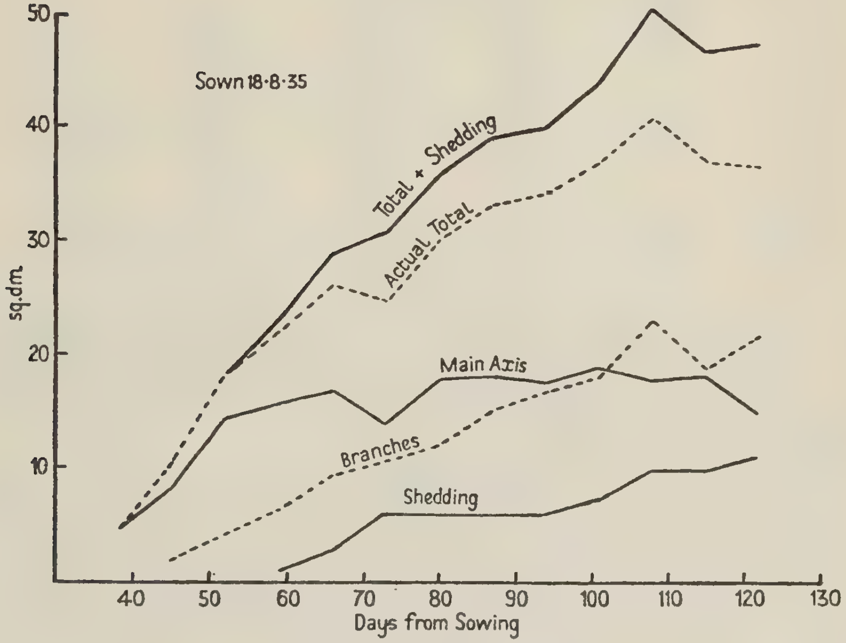

FIG. 2.

FIGS. 1 and 2. Fig. 1. Graph showing maximal area and dry weight (gm. per 100 sq. cm.) for successive leaves up to the end of December 1935. Fig. 2. Graph showing the increase in total leaf surface of the plant in season 1935–6, the amounts contributed to this total by the different classes of leaves, and the extent of the main axis shedding.

Both the maximal leaf surface of the plant and the time at which it is reached are profoundly influenced by seasonal factors, and in fact provide one method by which the extent of these seasonal fluctuations may be estimated. Thus, to take only one example, the maximal area recorded in 1934–5 was 3,743 sq. cm. on 30.10.34, and for a similar date of sowing in 1935–6 was 4,086 sq. cm. on 4.12.35. Both these were 'good growing seasons' so that in a poor year the total leaf surface produced would be very much less than either of the above values.

(d) *Change with age. Increase in thickness of lamina.*

Although the full expansion of the leaf lamina is soon attained (compare Table I), other changes continue as the leaf ages. The texture becomes harsher and more leathery and there is an actual increase in thickness. The ratio of dry weight per 100 sq. cm. of leaf surface forms a good measure of these changes: values of this ratio for the same leaves as in Table I are presented in Table II, inspection of which shows that this value is continually increasing with time.

TABLE II

Dry Weight (gm.) per 100 sq. cm. of Leaf Surface

Date recorded.	Leaf No. 5.	Leaf No. 10.	Leaf No. 15.	Leaf No. 20.	Leaf No. 25.
25.9.35	0·588	0·579	—	—	—
2.10.35	0·604	0·583	—	—	—
9.10.35	0·663	0·622	0·569	—	—
16.10.35	0·607	0·607	0·636	0·794	—
23.10.35	Shed	0·627	0·712	0·778	—
30.10.35	—	0·664	0·748	0·755	—
6.11.35	—	0·776	0·764	0·892	—
13.11.35	—	0·702	0·792	0·986	0·865
20.11.35	—	0·746	0·912	0·992	0·883
27.11.35	—	Shed	0·872	1·015	1·000
4.12.35	—	—	0·862	1·052	1·065
11.12.35	—	—	0·862	1·043	1·052
18.12.35	—	—	0·952	1·140	1·048
25.12.35	—	—	—	—	—
1.1.36	—	—	1·020	1·230	1·165

The effect observed is actually separable into two parts. Firstly, there is the continued increase in thickness of the individual leaf with age, and, secondly, the progressive increase in thickness of successively produced leaves, as can be seen from the second curve of Fig. 1. Consideration of the causal factors at work is, however, difficult, since it is almost impossible with the present limited data to separate the different effects of (1) nodal position, (2) time, i.e., ageing *per se*, and (3) seasonal climatic changes. Definite proof of the existence of this third factor is furnished by the results of the 1934–5 experiment, illustrated graphically in Fig. 3, which show the average thickness of the leaves of the later-sown cotton to be much the greater.

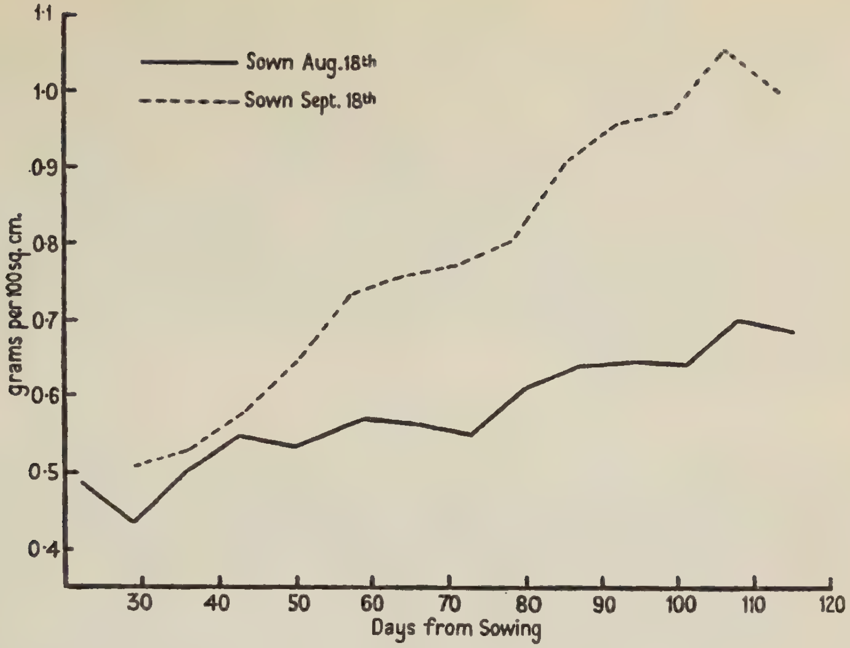

FIG. 3.

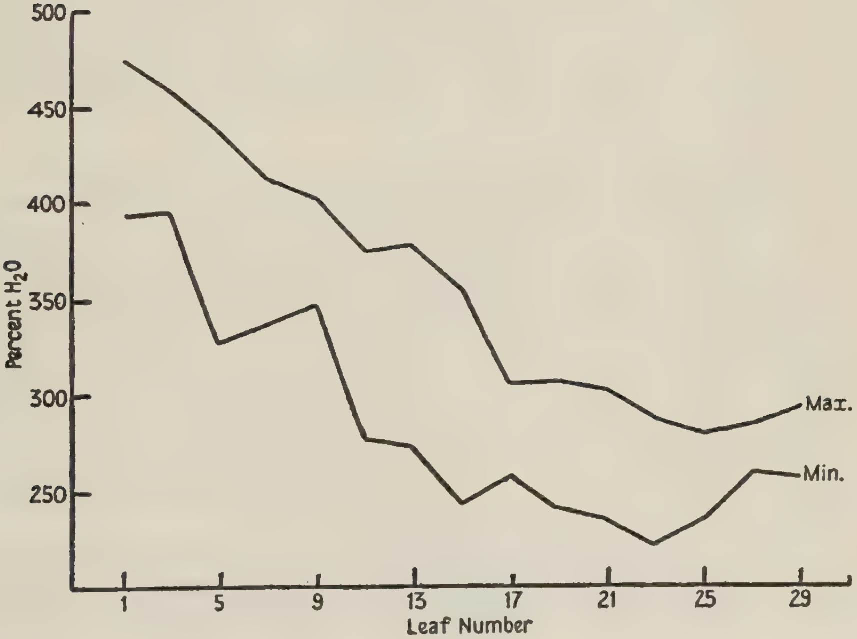

FIG. 4.

FIGS. 3 and 4. Fig. 3. Graph showing the increase, with time, of average dry weight (gm.) per 100 sq. cm. of leaf, for two sowing dates; season 1934–5. Fig. 4. Graph showing the maximal and minimal water contents recorded per leaf up to the end of the main growing period in season 1935–6.

At the same time it is clear that the changes with age or time in a single leaf are not closely related to the differences found between successive leaves at any one sampling date. The true change with time in all leaves is a continued increase, as may be seen by reading the individual columns in Table II from top to bottom. The apparent change deduced from the leaf series at any time is obtained by reading the individual rows from right to left: while the weight per unit area rises with leaves of increasing age for the first three sampling dates, reflecting the true effect; at all later times no such resemblance is found to the actual changes. In fact, the age sequence so deduced would be either a continuous fall or else a slight rise during early leaf history followed by a rapid fall.

IV. WATER CONTENT

Changes in the water content of cotton leaves appear to be of three distinct types. Of these, the best known are the fluctuations from hour to hour throughout the day as a consequence of varying degrees of 'waterstrain'. Secondly, there are the longer period fluctuations in the *basic water content* of the leaf correlated with changes in the water supplying power of the soil consequent upon irrigation. These are mentioned by Hawkins (1927), but no details for any particular leaf are given. Thirdly, and as far as the author is aware almost entirely uninvestigated, there are the various drifts occasioned by the whole complex of factors referred to in the preceding section. These major changes must necessarily provide a basis on which all the other changes (described above as fluctuations) are to be compared, and will therefore be considered first.

(a) *Major changes. Regular drifts with time.*

Observations of the water content of the odd-number main axis leaves were carried out weekly during 1935–6, at similar times each day. As in the case of the dry weight data, a double series of changes is found to take place. Fig. 4 shows the maximum and minimum water content,[1] expressed on a dry weight basis, observed in any leaf up to the end of the main growing period. The falling water content from leaf to leaf is very well marked up to about the 15th or 17th node, after which it tends to fluctuate between more or less constant limits (310 and 220 per cent.).

Besides this difference in successive leaves, there is also a fall in the moisture content of the individual leaf with time. The extent of this regular age drift is illustrated in Fig. 5 for leaves of numbers 7, 15, and 21.

Both sets of changes follow closely those previously demonstrated for dry weight per 100 sq. cm., and are, no doubt, intimately connected: thus, an increase in dry weight is accompanied by a decrease in the water content of the leaf.

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[1] These two extremes are given in preference to an average value, since, owing to climatic changes and the varying periods during which the different leaves remain attached to the plant, it is impossible to obtain a true estimate of this average.

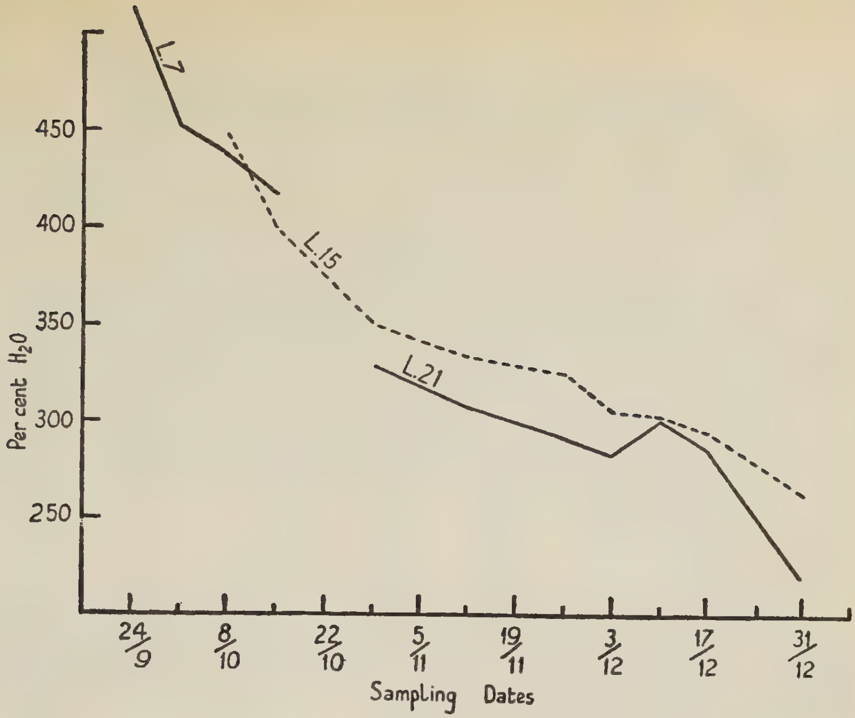

FIG. 5.

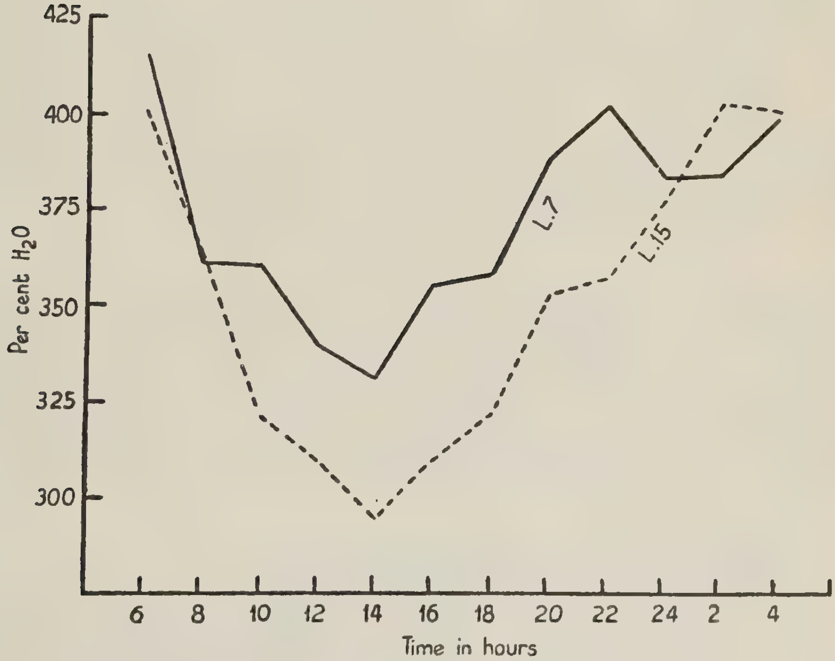

FIG. 6.

FIGS. 5 and 6. Fig. 5. Graph showing changes, with time, of the water content of leaves numbered 7, 15, and 21; season 1935–6. All observations at 6·00 hours. Fig. 6. Graph showing the average percentage of water at 2-hourly periods in leaves numbered 7 and 15 over 24 hours from October 15 to 16, 1935.

(b) *Diurnal fluctuations. 'Waterstrain.'*

Fig. 6 illustrates the nature of the daily fluctuations in leaf water content occurring in the Gezira. The data are for October 15–16, 1935. Starting at a high level, the percentage of water in the leaf falls continuously as the day advances and conditions become hotter and more desiccating and the water supply to the leaf becomes less and less able to keep pace with transpiration losses. In the neighbourhood of 14·00 hours a minimum value is reached and recovery begins. The number of hours required for this recovery to be complete has been found to depend on the extent of the water deficit to be made up. Further, it has been shown by Crowther (1932) that changes in this deficit or 'waterstrain' are independent of normal irrigation and depend solely on climatic changes.

(c) *Effect of irrigation.*

Although the normal fourteen-day irrigation, as practised in the Gezira, has little influence on the daily water deficit, it shows a marked effect on what has been termed the basic water content of the leaf. This can be seen clearly in Fig. 7, which shows for leaf number 15 the maximum and minimum moisture contents recorded during the day.

Observations were taken (1) at watering, i.e. when the water supplying power of the soil was at its lowest, and (2) seven days later, when it could be assumed that the irrigation water had penetrated to the subsoil and that the water supplying power of the soil was consequently high. A large increase in water content following on irrigation is readily observable throughout the whole of the period under consideration, although it is slightly masked at the beginning by the fall due to the age drift. There appears to be no correlation between the degree of 'waterstrain' and the time of irrigation.

V. NITROGEN CONTENT

All nitrogen was estimated by the usual Kjeldahl method. No special modification of the method for the reduction of the nitrate nitrogen was employed, since it may safely be assumed that there is sufficient organic reducing material present in a sample of this nature to cause this reduction to take place without assistance. The figures given in the subsequent sections, therefore, represent all nitrogen present, irrespective of the particular form in which it occurs in the plant.

(a) *Nitrogen as a percentage of the dry weight.*

Table III shows the average nitrogen content of the various leaves, expressed as a percentage of the dry weight, at successive four-weekly intervals throughout the season 1935–6. Actually, sampling was carried out weekly, but the figures given here will be sufficient to illustrate the changes taking place.

On examining the table it is evident that *at any one time* the percentage of nitrogen is highest in the youngest leaf present and lowest in the oldest. This is the usual relation found by many workers and is so often assumed to be an expression of the fundamental differences between successive leaves.

TABLE III

Leaf Nitrogen expressed as a Percentage of the Dry Weight

Leaf No.	26.9.35	24.10.35	21.11.35	19.12.35	23.1.36*	20.2.36	19.3.36
1	*3·28*	Shed	—	—	—	—	—
3	3·52	”	—	—	—	—	—
5	4·06	”	—	—	—	—	—
7	4·62	*2·62*	Shed	—	—	—	—
9	5·14	3·04	”	—	—	—	—
11	*5·74*	3·38	*2·38*	Shed	—	—	—
13	—	3·60	2·54	”	—	—	—
15	—	3·68	2·66	*2·30*	Shed	—	—
17	—	3·50	2·66	2·28	*2·12*	*1·90*	Shed
19	—	4·00	2·84	2·42	1·98	2·00	”
21	—	*4·10*	2·92	2·66	2·06	2·10	”
23	—	—	3·14	2·62	2·10	1·98	”
25	—	—	*3·34*	2·90	2·42	2·32	*2·06*
27	—	—	—	2·86	2·58	2·72	2·36
29	—	—	—	*2·96*	2·74	3·12	2·72
31	—	—	—	—	*3·38*	3·32	2·96
33	—	—	—	—	—	3·40	3·30
35	—	—	—	—	—	*3·22*	3·22
37	—	—	—	—	—	—	*3·44*

* 5 weeks' interval.

Further consideration of the table shows this conclusion to be entirely erroneous. The result is due solely to the difference in age of the leaves compared; if the history of any one leaf is followed across the table it will be seen that its nitrogen content falls continually with time until the leaf is shed. The only leaves at all comparable between successive samples as regards physiological age are (*a*) the youngest and (*b*) the oldest leaves actually present on the plant at the time of sampling. The percentage nitrogen in these two groups is shown graphically in Fig. 8. The true relation between the various leaves is now evident. *Age for age*, during the whole of the main growing period, it is the earliest produced leaf that has the highest nitrogen content; this is the exact opposite of the relationship deduced from the result of a single sample.

Leaves from number 31 onwards, which were produced later during the secondary growing period, exhibit a slight rise in nitrogen content. This result is in conformity with previous work on the nitrogen metabolism of the cotton plant (Crowther, 1934) and has been shown to be a consequence of the resumption of nitrogen uptake by the roots after maturation of the first crop of bolls has been completed.

The results generally fall into line with those previously presented for basic water content, except that no regular diurnal fluctuations or changes consequent upon irrigation have been observed.

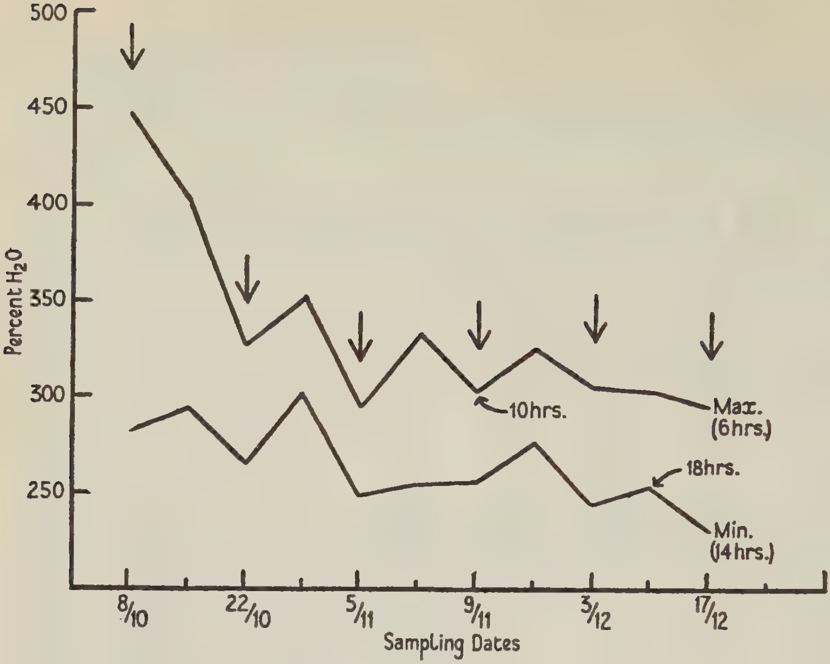

FIG. 7.

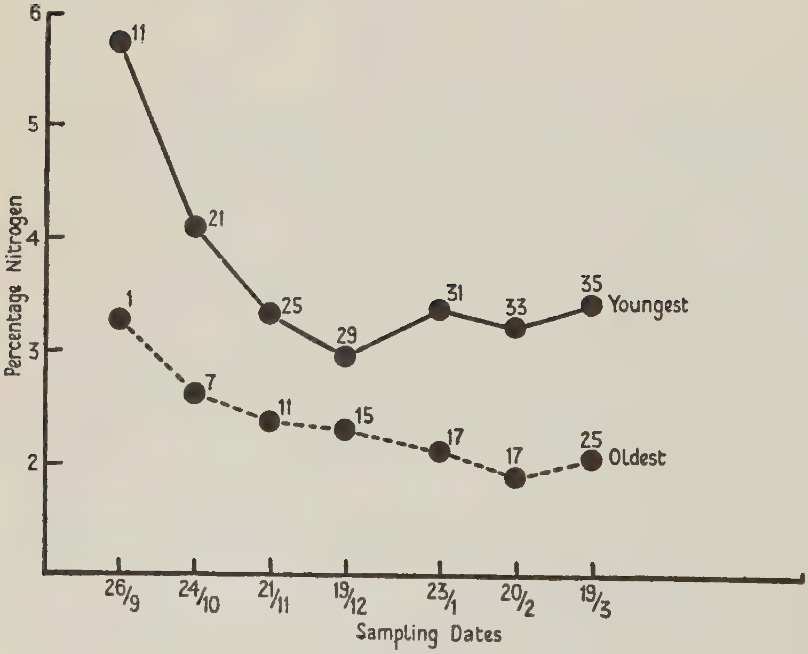

FIG. 8.

FIGS. 7 and 8. Fig. 7. Graph showing changes with time of the maximal and minimal water content of leaf number 15, for season 1935–6. Irrigation dates are indicated by arrows. Fig. 8. Graph showing the percentage of nitrogen in the youngest and oldest leaves present on the plant at the time of sampling.

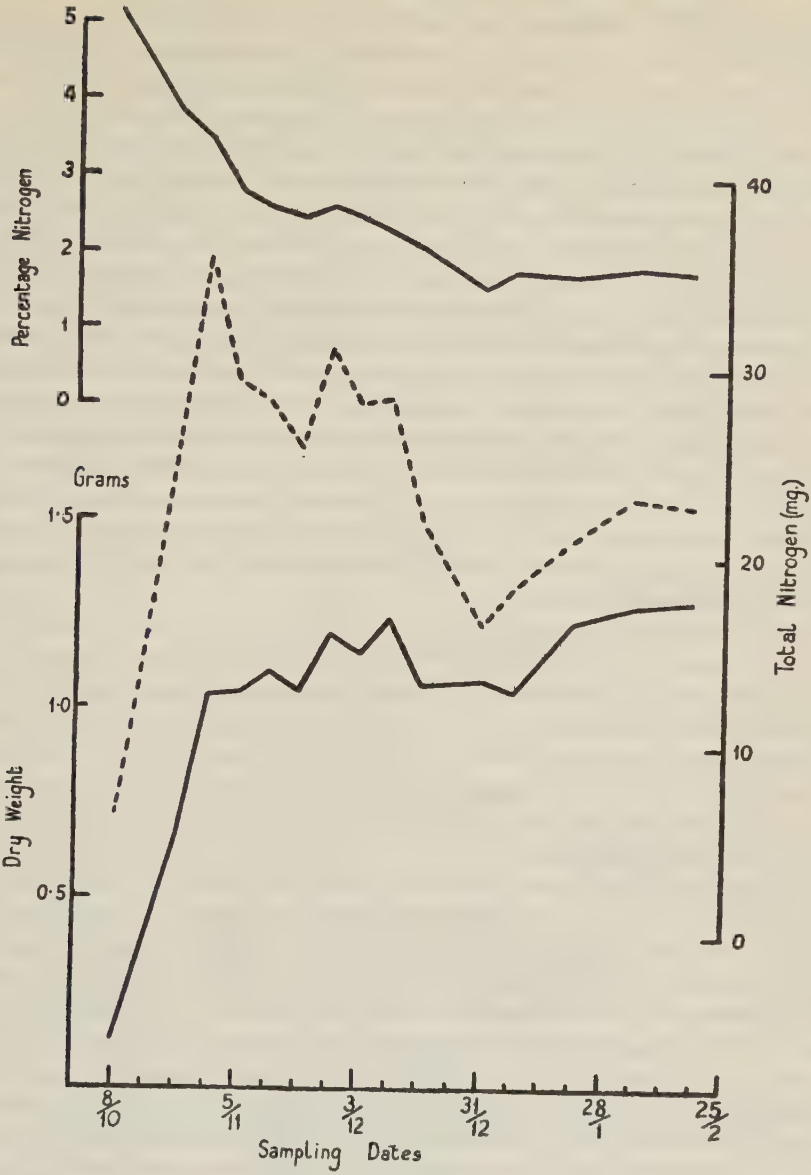

FIG. 9. Graph showing changes with time of the dry weight, total and percentage nitrogen of leaf number 15 in season 1935–6.

(b) *Changes in total nitrogen.*

Owing to the importance of nitrogen supply in the metabolism of the plant it will be of interest to trace the changes in the total amount of nitrogen present in the leaves. As these changes follow a similar course in all the leaves examined it will be sufficient to follow the history of a single leaf in detail, as it grows and ages. Fig. 9 shows the total nitrogen in leaf number 15 plotted against time. Percentage nitrogen and leaf dry weight curves are also shown on this figure, plotted on the same time scale for comparison.

It will be seen that, contrasted with percentage nitrogen which falls steadily from the beginning, the total amount of nitrogen in the leaf rises rapidly to a maximum and then falls off. This maximum corresponds to the time at which complete expansion of the lamina is reached, after which the total nitrogen falls in conformity with the percentage nitrogen content. As was only to be expected, there is much more irregularity exhibited by the total nitrogen than by the percentage nitrogen curve. This, however, is mainly due to sampling differences as is evident from the close way in which fluctuations in the former curve follow fluctuations in the dry weight of the sample.

Although, as stated above, the total nitrogen curves all have approximately the same shape in different leaves, it must be borne in mind that the levels of these curves will not all be the same. The total amount of nitrogen present in the leaf depends on the dry weight and the percentage of nitrogen present in that dry weight, and it has already been proved that both of these vary considerably from leaf to leaf.

VI. Conclusions and Summary

1. Plants of X1530, a type of Sakellaridis cotton, were grown under irrigation during the two seasons 1934–5 and 1935–6 and the results of observations on area, dry weight, water content, and nitrogen content of the leaves are reported. All leaf samples were grouped according to the nodal positions of the different leaves so that changes with time in the successive individual leaves could be determined.

2. Attention is first drawn to the very different shapes and sizes attained by the successive leaves as an indication that there may be also similar differences in their more fundamental characteristics. This is actually found to be the case in respect of all those features examined. Not only do no two successive leaves commence their life history with the same constitution, but the course of the subsequent changes taking place as they age may be also dissimilar. As pointed out by Richards (1934) we are dealing here with two distinct age drifts; that of the individual leaf and that of the shoot as a whole. Attempts to deduce the age sequence of a single leaf from the particular values observed in successive members at any one time are therefore invalid.

3. The value of the dry weight per 100 sq. cm. (leaf thickness) was found to continually increase with time in any one leaf, while the initial value of this ratio also became greater as successive leaves were produced by the shoot. Evidence is brought in support of the contention that the effect observed was, to some extent, the result of climatic changes.

4. Marked differences were found in what has been termed the basic water content of the leaf. Here the initial water content, expressed on a dry weight

basis, fell rapidly from leaf to leaf up to about the 15th node, after which it became very nearly constant. The water content of any one leaf was also found to fall rapidly as it aged.

5. Relations similar to the above were found to hold for percentage nitrogen. There was, however, a slight rise in the initial nitrogen levels of the leaves produced during the second growing period. This was to be expected, since it has been shown by Crowther (1934) to be a consequence of the resumption of nitrogen uptake by the roots after maturation of the first crop of bolls has been completed.

6. The total nitrogen in any particular leaf, as contrasted with the percentage nitrogen, rose rapidly to a maximum, which corresponded to the time at which the expansion of the lamina was completed, and then continued to fall slowly in conformity with the percentage content.

7. The extent of the diurnal fluctuations in leaf water content is indicated, while data illustrating the marked effect of irrigation on basic water content are also presented.

In conclusion, the author wishes to place on record his appreciation of the very able way in which his native technical assistant, Mohammed Effendi Ali Babikir, carried out the vast number of routine observations necessary, while grateful acknowledgement is made to the staff of the Chemical Analytical Section of this Service for undertaking the burden of nitrogen analysis.

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Studies in Flower Structure

III. On the 'Corona' and Androecium in Certain Amaryllidaceae.[1]

BY

AGNES ARBER

With four Figures in the Text

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I. INTRODUCTION

ALTHOUGH the nature of the 'corona' in the Amaryllidaceae has been debated for at least a century, no agreed conclusion seems to have been reached. In the present paper an attempt is made to see what light can be thrown upon the question by means of a comparative study of serial sections of the flowers of certain Pancratieae and Coronatae. Incidentally the vascular relation between a perianth member and its superposed stamen is considered.

In the division of the Amaryllidaceae into tribes, Baker's *Handbook* (1888) is followed.

II. THE 'CORONA'

(i) *Observations*

(a) *Pancratieae.*

Hymenocallis festalis. This plant is a hybrid, raised by Major Pam, between *H. calathina* Nich. and *Elisena longipetala* Lindl., which belong to related Peruvian genera. The flowers of the hybrid are very large and slightly zygomorphic. One of them (placed upright) is shown, reduced to one-third, in Fig. 1, A, p. 294. It will be seen that the filaments emerge from the edge of a conspicuous 'corona' or stamen-cup. The remaining drawings in Fig. 1 are

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[1] This paper represents part of the work carried out during the tenure of a Leverhulme Research Fellowship. I am indebted for material to Mr. E. A. Bowles, Major Albert Pam, the Lady Rockley, and Mr. W. T. Stearn.

from serial sections through a bud. B1 shows the structure at the level at which the stamen-cup is just becoming free from the perianth segments. In B2*a* it is completely detached, while in B3 it is cut just below its upper margin, where the filaments are becoming free; in these two figures the perianth is not shown. The filaments are massive, and each has a single large bundle.

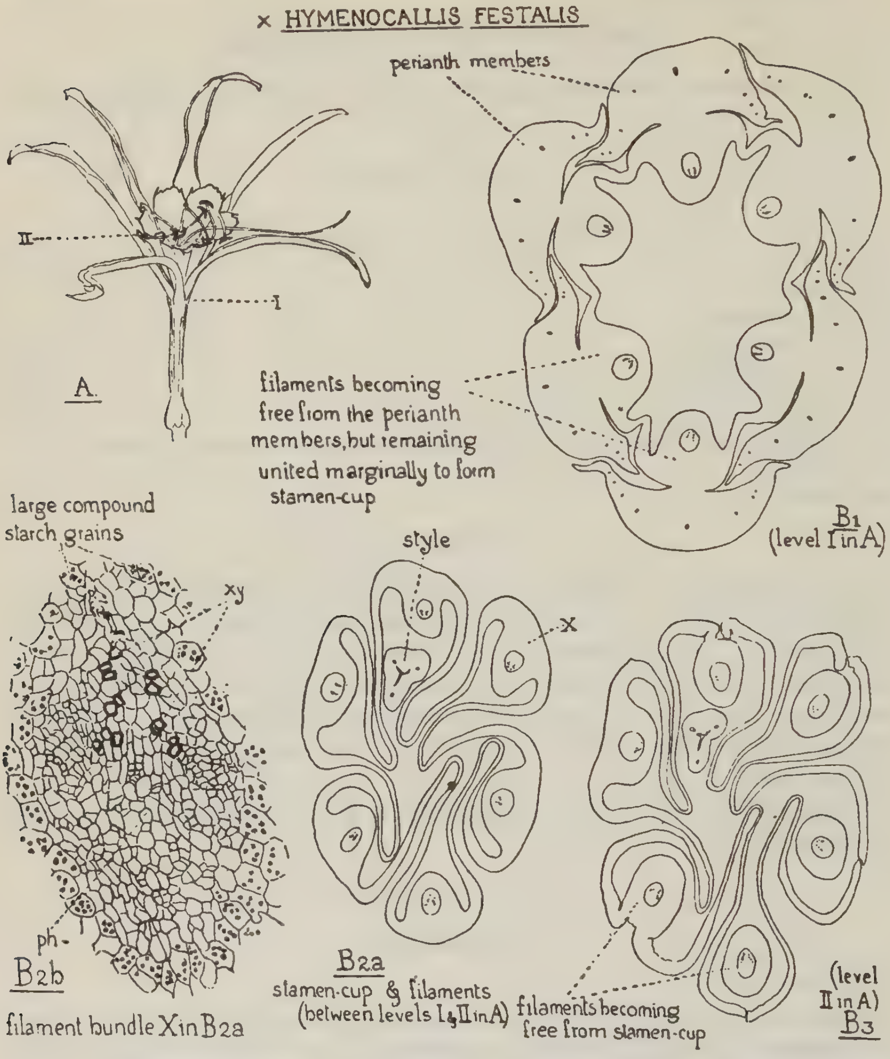
× HYMENOCALLIS FESTALIS
perianth members
II
I
A.
filaments becoming free from the perianth members, but remaining united marginally to form stamen-cup
B1 (level I in A)
large compound starch grains
xy
style
x
ph
B2b
filament bundle X in B2a
B2a
stamen-cup & filaments (between levels I & II in A)
filaments becoming free from stamen-cup
(level II in A)
B3

Fig. 1. ×*Hymenocallis festalis*, hybrid between *H. calathina* Nich. and *Elisena longipetala* Lindl. (Major Pam, May 2, 1935). A, flower (×⅓ *circa*). B1–B3, sections from a transverse series passing upwards from below through a bud. B1, B2*a*, B3 (×9 *circa*). B1, at level I in A, gynaeceum omitted. B2*a* and B3, sections through stamen-cup and style, perianth omitted. B3, at level II in A. B2*b*, filament X in B2*a* (×99 *circa*); starch grains are not shown in any other figure. Throughout the figures in this paper, *xy* = xylem, and *ph* = phloem.

One of these is shown in B2*b*; there are indications of radial arrangement of the elements, suggesting that some secondary development has taken place. The part of the stamen-cup between the filaments is entirely non-vascular.

Hymenocallis amancaes Nich. and *Pancratium illyricum* L. I have cut serial sections of a flower of each of these two species, grown by Major Pam. I find that the 'coronal' structure shows no essential difference from that described for *H. festalis*.

Eurycles Cunninghamii Ait. This species was introduced from North Queensland by Lady Rockley. The stamen-cup, which is shown laid open in Fig. 2, D, p. 296, is lobed and bifid between the filaments. Sections such as that drawn in Fig. 2, F, show that, in the origin of the stamen-cup and its relation to the perianth, this species closely resembles *Hymenocallis festalis*. A detail that may be mentioned, though it does not bear upon our present inquiry, is that the anther at its base completely enrings the filament (E), a condition which I have also noticed in one of the Gramineae, *Anomochloa marantoidea* Brongn. (Arber, 1929) and in one of the Capparidaceae, *Gynandropsis speciosa* DC.

Eustephia pamiana Stapf. This Argentine species (Stapf, 1929), of which I have examined material grown by Mr. Bowles, is included here because, although it has no stamen-cup, its free filaments are winged (Fig. 2, A, p. 296). B1 is a section through the flower at a level corresponding to the sections of other genera shown in Fig. 1, B1, p. 294, and Fig. 2, F.

(b) *Coronatae*.

In this tribe I have been able to examine only species belonging to the genus Narcissus.

Narcissus ? minor. Serial sections were cut through a bud of a garden form grown under this name. Fig. 3, A1, p. 297, passes through the perianth tube, which in A2 is beginning to separate into corona and perianth segments. Owing to a slight obliquity in the section, the level just below the separation is shown on the left-hand side, and it is seen that while still in the perianth tube, the bundles have divided to form two series, the outer destined for the perianth segments and the inner for the corona. The bud was so young that lignification was only just beginning in the corona and perianth segments, but here and there, in bundles which happen to be more advanced than the rest, it is seen that the coronal strands are not orientated normally, but have their xylem directed towards the xylem of the perianth bundles; in other words, the coronal bundles have dorsal xylem and ventral phloem, while the perianth bundles have, as usual, ventral xylem and dorsal phloem. This distinction is seen in Fig. 3, B. In Fig. 4, C1–C5, p. 299, from a series passing upwards from below, the origin of the two opposed bundles, *m'* and *m''* in Fig. 3, A1, p. 297, can be traced. The normally orientated strand (C1) widens (C2), and divides into two bundles, *m* and *n*, facing one another in a plane parallel to the surface of the perianth tube (C3). One of these, *m*,

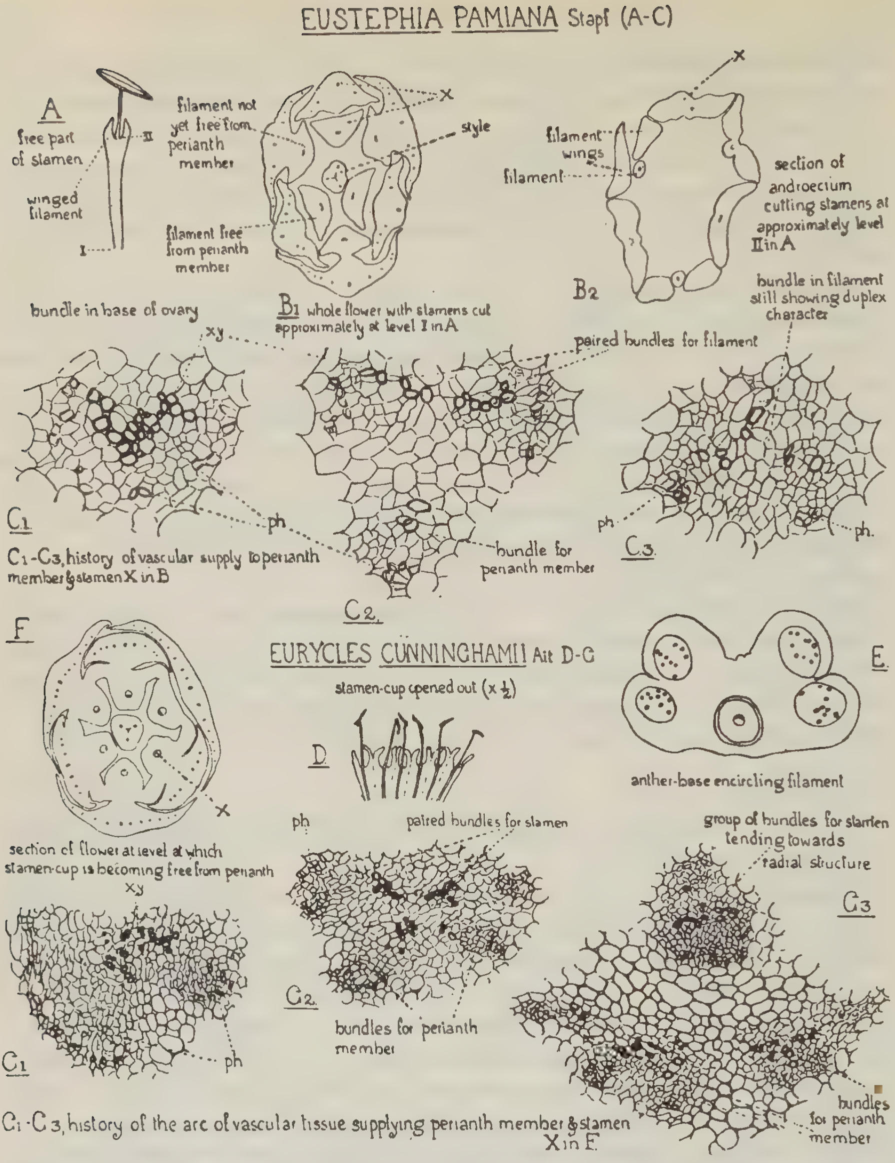
EUSTEPHIA PAMIANA Stapf (A-C)
A
free part of stamen
winged filament
I
II
filament not yet free from perianth member
filament free from perianth member
x
style
B1 whole flower with stamens cut approximately at level I in A
x
filament wings
filament
section of androecium cutting stamens at approximately level II in A
B2
bundle in base of ovary
xy
paired bundles for filament
bundle in filament still showing duplex character
C1
ph
C1-C3, history of vascular supply to perianth member & stamen X in B
bundle for perianth member
C2
ph
C3
ph.
F
EURYCLES CUNNINGHAMII Ait D-G
stamen-cup opened out (x ½)
D
E
anther-base encircling filament
x
section of flower at level at which stamen-cup is becoming free from perianth
xy
ph
paired bundles for stamen
group of bundles for stamen tending towards radial structure
G3
G2
bundles for perianth member
G1
ph
G1-G3, history of the arc of vascular tissue supplying perianth member & stamen X in F
bundles for perianth member

FIG. 2. *Eustephia pamiana* Stapf (Mr. E. A. Bowles, April 22, 1936). A, free part of one stamen (nat. size, *circa*). B1 and B2, sections from series through a young flower (× 14); B1, cutting stamens at approximately the level of I in A; B2, showing androecium only, approximately at level II in A. C1–C3, sections (× 193 *circa*) from a series from below upwards, to show the history of the bundle supplying stamen and perianth member X in B1. These diagrams are orientated differently from B1, so as to bring the adaxial faces uppermost. C1, in base of ovary; C2, in top of ovary; C3, in free part of filament just above level of B1. D–G, *Eurycles Cunninghamii* Ait., from a plant exhibited at the Roy. Hort. Soc. by Lady Rockley, June 5, 1935. D, stamen-cup opened out (× ½); E, transverse section of a stamen near the base of the anther (× 23); F, transverse section through flower at level at which the stamen-cup is being detached from the perianth members (× 7). G1–G3, sections (× 77) from a series from below upwards to show the history of the vascular supply for stamen and perianth member X in F. G1, from a section at base of gynaeceum below ovary cavities, to show arc of vascular tissue shortly above its emergence from the vascular ring of the pedicel; G2, at a higher level in the ovary wall; G3, in the perianth tube.

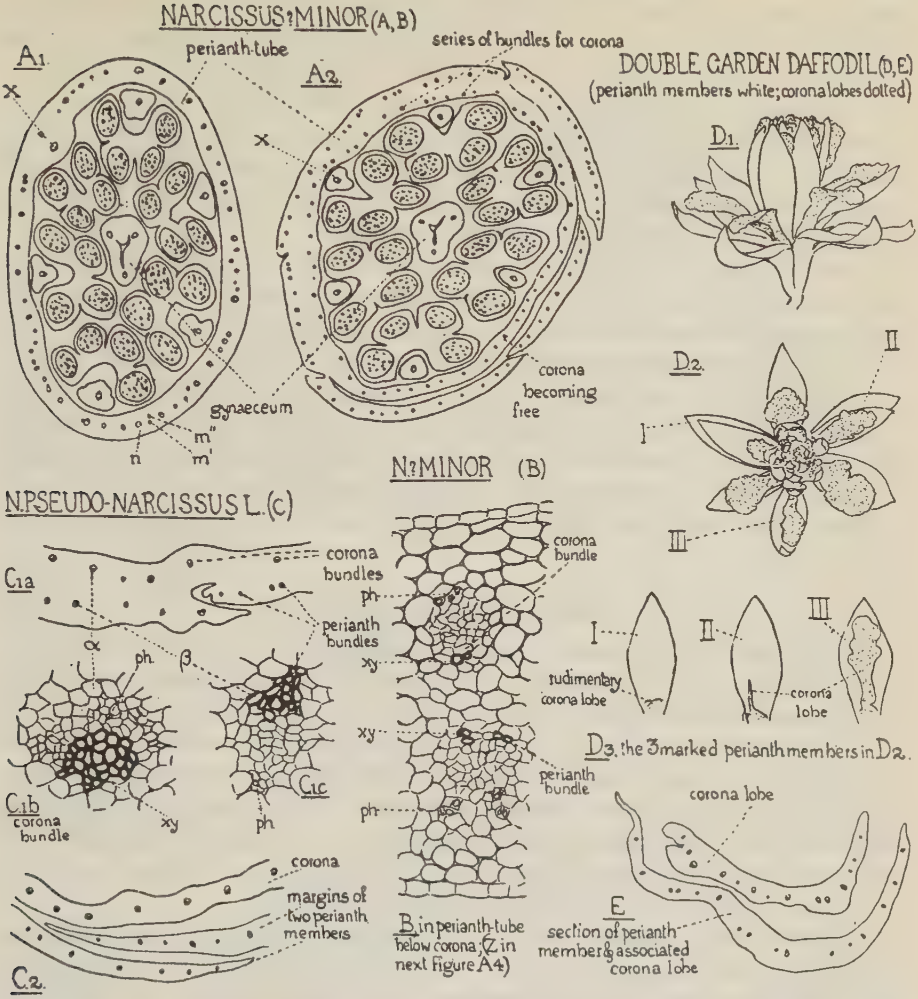
NARCISSUS ? MINOR (A, B)
A1
X
perianth-tube
A2
series of bundles for corona
x
gynaeceum
m''
n
m'
corona becoming free
DOUBLE GARDEN DAFFODIL (D, E)
(perianth members white; corona lobes dotted)
D1
D2
I
II
III
N ? MINOR (B)
corona bundle
ph
xy
xy
perianth bundle
ph
B, in perianth-tube below corona; (Z in next Figure A4)
N. PSEUDO-NARCISSUS L. (C)
C1a
corona bundles
perianth bundles
α
ph.
β
C1b
corona bundle
xy
ph
C1c
corona
margins of two perianth members
C2
I
II
III
rudimentary corona lobe
corona lobe
D3. the 3 marked perianth members in D2.
corona lobe
E
section of perianth member & associated corona lobe

FIG. 3. A, B, *Narcissus ? minor*. A1 and A2, transverse sections through the perianth tube of a bud below and just at the level of the detachment of the corona (× 14). The origin of the perianth and stamen bundle X is shown in Fig. 4, A1–A5, p. 299, and of *n*, *m'*, and *m''*, in Fig. 4, C1–C5. B, the two bundles in the perianth tube marked Z in Fig. 4, A4 (× 193 *circa*). C, *N. pseudo-narcissus* L. (wild form). C1*a*, segment of top of perianth tube showing two series of bundles, one of which is normally orientated for perianth, while the other is inverted for corona (× 14). Two of these bundles, *α*, corona bundle, and *β*, perianth bundle, are drawn (× 193 *circa*) in C1*b* and C1*c*. C2 (× 14) at a level above C1*a*, to show the separation of the perianth segments and corona, which are still united in C1*a*. D, double daffodil (garden form); D1–D3 (× ½); perianth white, corona lobes, dotted. D1 and D2, flower from the side and above. D3, the three outer perianth segments with their associated corona lobes (× ½). E, transverse section of a perianth segment and associated corona lobe (× 14).

divides into two, *m'* and *m''*, in this plane (C4), and the phloems move round until the bundles face one another (C5).

Narcissus pseudo-narcissus L. Since the bud of *N. ? minor* described above was too young to show full lignification, I have added sections through the flower of a wild daffodil, in which the bundles were completely developed, so that the orientation of the coronal strands is clear. Fig. 3, C1*a*, shows the structure of a small part of the upper region of the perianth tube; the overlapping margins of two perianth segments are seen just becoming free from the tube. In C1*b* and C1*c*, a coronal bundle, α, and a perianth bundle, β, are drawn on a larger scale. It will be noticed that the inverted coronal bundle has more massive xylem than the perianth bundle.

The behaviour of the corona in double forms of *N. pseudo-narcissus* is of some interest. There is much variation in structure among these flowers, but in characteristic examples there is generally no 'trumpet', and each of the numerous perianth segments bears on its inner face a separate lobe, exactly resembling the corona of the normal form in colour and texture (Fig. 3, D1–D3). Serial sections through these perianth segments, with their associated lobes (Fig. 3, E), reveal the normal orientation of the bundles in the perianth segments, but, in the corona lobes, the reversed position and stronger lignification which have been already noticed in the corresponding parts of the single flower.

(ii) *The interpretation of the 'corona'*

It would serve little purpose to enter in detail into the history of the earliest opinions about the corona; but as examples of views that have been held, it may be mentioned that the corona of Narcissus was described by Doell in 1857 as comparable with the ligules of certain foliage leaves, by Masters in 1865 as consisting of modified stamens, and by Smith in 1866 as composed of confluent petal stipules. Most of the earlier writers dealt with Narcissus only; and those who brought any member of the Pancratieae into the argument seem always to have assumed that the staminal cup of this tribe must be explained on the same lines as the corona of Narcissus.

In 1875 Eichler reviewed the subject and came to the conclusion that the corona was not staminal, but was an outgrowth of the perianth, both in Narcissus (Coronatae) and in members of the Pancratieae. On the other hand, in 1888 Pax interpreted the corona of the Pancratieae as staminal stipules, and regarded that of the Coronatae as a corresponding structure, which had, however, become ligular and free from the filaments. Ten years later Čelakovský (1898) concluded, like his predecessors, that the corona in the Pancratieae and in the Coronatae is of the same nature.

Coming to the present century, we find that Velenovský in 1910 supported the view that the corona was always of the nature of fused staminal stipules, while, as lately as 1933, the same conclusion is maintained in the most recent edition of Goebel's 'Organographie', in which a hypothetical diagram is given (Fig. 1951, p. 1865) tracing the assumed development of the free corona of Narcissus from the stamen-cup of the Pancratieae.

It is remarkable that the numerous botanists who have approached the

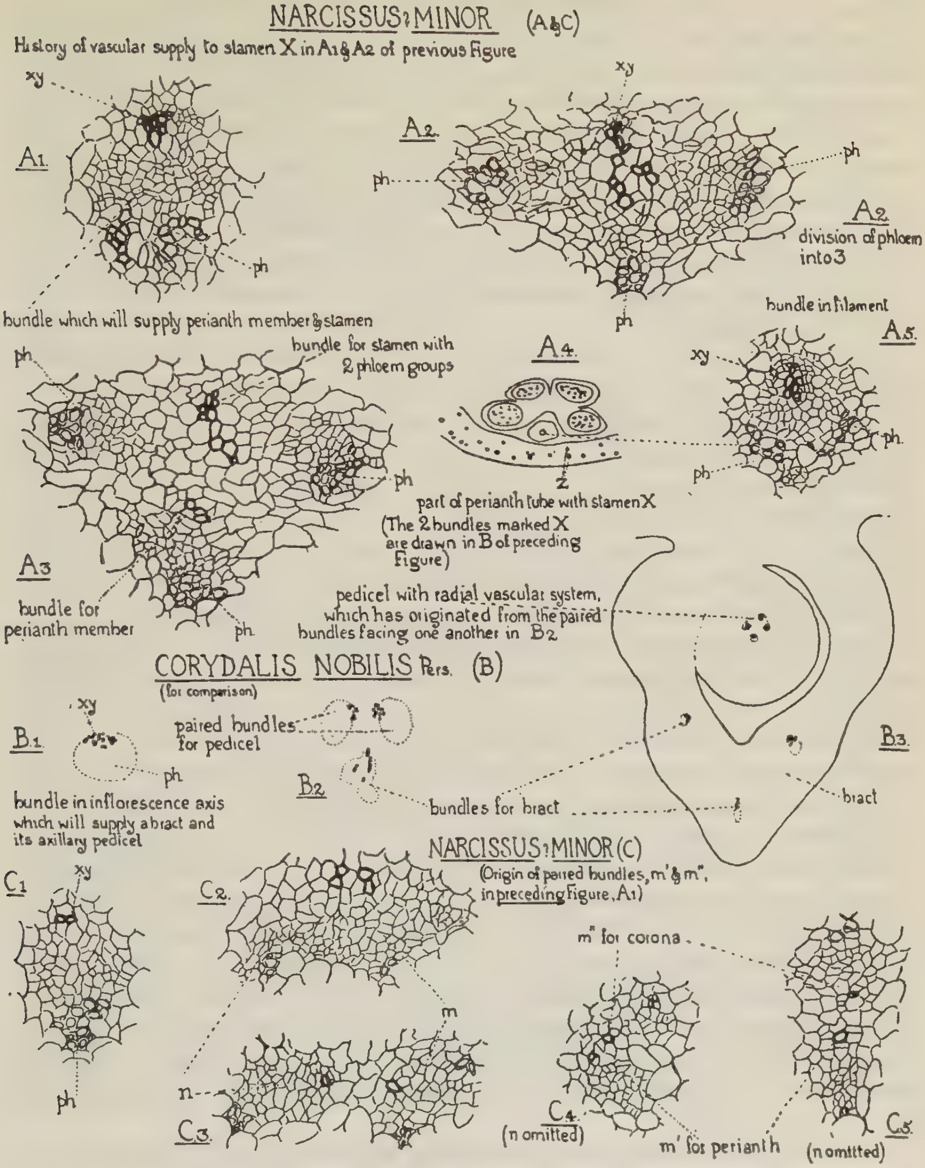
NARCISSUS ? MINOR (A & C)
History of vascular supply to stamen X in A1 & A2 of previous Figure
xy
A1.
ph
bundle which will supply perianth member & stamen
A2.
xy
ph
ph
A2
division of phloem into 3
ph
bundle in filament
A5.
xy
ph
ph
A4.
a
z
part of perianth tube with stamen X
(The 2 bundles marked X are drawn in B of preceding Figure)
ph
bundle for stamen with 2 phloem groups
ph
A3
bundle for perianth member
ph
pedicel with radial vascular system, which has originated from the paired bundles facing one another in B2
CORYDALIS NOBILIS Pers. (B)
(for comparison)
xy
B1.
ph
paired bundles for pedicel
bundle in inflorescence axis which will supply a bract and its axillary pedicel
B2
bundles for bract
bract
B3.
NARCISSUS ? MINOR (C)
(Origin of paired bundles, m' & m", in preceding Figure, A1)
C1
xy
ph
C2.
m
n
C3.
m" for corona
C4.
(n omitted)
m' for perianth
C5.
(n omitted)

FIG. 4. A and C, *Narcissus ? minor*. A1–A5, from a series of sections from below upwards, tracing the history of the vascular supply to stamen X in Fig. 3, A1, A2. A1–A3, A5 (× 193 *circa*); A4 (× 14). B, *Corydalis nobilis* Pers. B1–B3, transverse sections (× 47) from a series from below upwards through an inflorescence axis showing the origin of the vascular system for a bract and pedicel; xylem elements alone indicated. (A bundle supplying the keel of the bract, which is merely a branch of the median bundle, is not represented.) C, *Narcissus ? minor*. C1–C5, sections from a series from below upwards (× 193 *circa*) through the perianth tube below Fig. 3, A1, p. 297, to show origin of bundles *n*, *m'*, and *m"*; in C4 and C5, *n* is omitted.

subject from the *morphological* angle, however their explanations have varied in other respects, have almost all agreed in thinking that the 'coronas' in the Pancratieae and in the Coronatae belong to the same category. Certain botanists who have been concerned with the Amaryllidaceae from the *taxonomic* standpoint have, however, thought differently. Baker (1888) in his account of this family describes the Coronatae (Narcissus tribe) as having a corona, while the distinguishing mark of the Pancratieae is 'Corona none. Stamens appendiculate towards the base, often united in a distinct cup.' Hutchinson (1934), also, makes a sharp separation between the 'true' corona of Narcissus and related genera, and the 'false' corona met with elsewhere in the Amaryllidaceae.

We have now to consider the bearing of the observations detailed in the present paper upon the theories which have just been briefly summarized. I think it will be recognized that, so far as the Pancratieae are concerned, these observations (Figs. 1, p. 294, 2, p. 296) serve to confirm the view that the stamen-cup of this tribe consists of staminal appendages. The free but winged filaments of *Eustephia pamiana* (Fig. 2, A, B1, B2) may be regarded as a first step in this direction. It is possible that there may be a correlation between the power of developing staminal wings and the unusually massive character of the filament bundle in the Pancratieae (e.g. Fig. 1, B2*b*, p. 294). The stamen-cup of *Eurycles Cunninghamii* may be treated as illustrating the next phase after the *Eustephia pamiana* type. The Eurycles-form definitely suggests non-separation of winged filaments; their junctions, indicated by broken lines in the drawing, form slight salients (Fig. 2, D, p. 296). A more advanced stage is reached in the large stamen-cup of *Hymenocallis festalis* (Fig. 1, A, p. 294), which is no longer easily analysable into components. The question whether winged stamens do not, or do, develop in basal union, and thus form a stamen-cup, may perhaps depend upon the degree to which the origin of the two whorls synchronizes. It is not possible to generalize from my observations, since I was only able to cut series from one flower each of *Hymenocallis festalis* (Fig. 1, B1, p. 294), *Eurycles Cunninghamii* (Fig. 2, F, p. 296) and *Pancratium illyricum*, but it is suggestive that in each of these flowers I found the stamens of the two whorls exserted at practically the same level, whereas in *Eustephia pamiana*, in which the winged filaments are separate, there was a detectable difference of level between the two whorls (Fig. 2, B1, p. 296).

In those genera belonging to the Pancratieae which I have examined, the stamen-cup is not only uniform in its obvious derivation from the stamens, it is also uniformly non-vascular, except for the single massive bundle belonging to each filament.

When we turn to Narcissus (Figs. 3, p. 297, 4, p. 299) we find that the corona here is so different in origin and structure from the stamen-cup of the Pancratieae that it is difficult to understand why these two structures have so often been treated as closely comparable. The corona of Narcissus has no connexion with the stamens,[1] but is split off from the inner surface of the perianth tube after the exsertion of the filaments (Fig. 3, A1, A2, p. 297). Its close association with the perianth is shown by the fact that in the typical double daffodil, in which no tubular corona is found, there is a strong tendency for each of the numerous free perianth segments to bear its own coronal lobe (Fig. 3, D). In contrast to the stamen-cup of the Pancratieae, the corona tends to be richly vascular; it is traversed by numerous bundles, which are not orientated like those of normal floral members, but are so placed that their xylems point towards the xylems of the perianth segments (Fig. 3, C and E, and Fig. 4, C5). The inverted orientation of the coronal bundles was recognized and figured by van Tieghem as long ago as 1871 (pp. 116–17, Pl. IV, 128–9), but it has seldom received any notice in subsequent discussions. It is, however, a fact of definite significance, involving, as it does, a basic architectural distinction, which must invalidate any close comparison between the corona of Narcissus and the stamen-cup of the Pancratieae. It confirms the conclusion which, as we have noticed, has already been reached by certain taxonomists, that the stamen-cup of the Pancratieae and the corona of Narcissus are fundamentally different. It is true that there is a certain superficial resemblance between these two structures, but this resemblance may well be due to parallelism in growth physiology. They both make their appearance late in the ontogeny of the flower (Baillon, 1860; Church, 1908), arising through meristematic activity affecting adjacent zones. Both stamen-cup and corona may be considered as secondary expressions of that tendency to tubular development, which finds its primary expression in the formation of the perianth tube.

If, then, we accept the staminal theory for the pseudo-corona of the Pancratieae (Hymenocallis, &c.), but reject it for the corona of the Coronatae (Narcissus, &c.) we have still to ask whether any homologue for the latter structure can be found. It seems to me that the nearest comparison, which can be suggested, is with the abnormal enations which sometimes give rise to the double-bladed type of foliage-leaf (Velenovský, 1907, pp. 409–10, Fig. 260; Worsdell, 1915; Ghose, 1923). In such enations the extra blade is so placed as to face the normal blade, as the corona of Narcissus faces the perianth. The nectary-scale of Ranunculus may also be an enation of the same type (Arber, 1936). In a later paper I hope to consider other outgrowths from petals, some of which may fall into the same category.

III. THE VASCULAR RELATION OF A PERIANTH MEMBER AND ITS SUPERPOSED STAMEN

In those Amaryllidaceae, which I have examined, a single bundle, or a small arc of vascular tissue, supplies the vascular system both for a perianth member and a stamen. That is to say, a collateral bundle branches into two collateral bundles with the same orientation, lying one within the other on the same

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[1] This statement is not intended to preclude the theory that the perianth members are themselves the homologues of stamens.

radius. It is obvious that this cannot be achieved by any straightforward process of division. The methods actually adopted show an interesting series of variations on one theme, which may be expressed in general terms as the division of the bundle into a median and two lateral strands, the median supplying the perianth segment, and the laterals, facing one another to right and left, supplying the stamen. The type of filament anatomy produced depends upon the degree to which these two bundles come into union. The division process is seen for *Eustephia pamiana* in Fig. 2, C1–C3, p. 296. In C2 two quite separate bundles are destined for the stamen; these bundles fuse in passing up, but in the filament they still show signs of a duplex character (C3). In *Narcissus ? minor* essentially the same scheme is followed, but, in the example figured, the supply for the stamen is a double bundle rather than two distinct bundles (Fig. 4, A3, p. 299). In another family, the Fumariaceae, an example may be found (*Hypecoum leptocarpum* Hook. f. et Thoms.) in which the discreteness of the two stamen strands, instead of being reduced, as in *Narcissus ? minor*, is so complete that the filaments remain permanently two-bundled (Arber, 1932, Fig. 6, p. 155). This has sometimes been interpreted as a sign that congenital fusion of two stamens has occurred, but an examination of the origin of the stamen supply shows that the two-bundled character is, in reality, only an extreme case of the particular type of bundle branching associated with superposition of a stamen upon a perianth member.

I have drawn attention elsewhere (Arber, 1932) to the resemblance of this mode of bundle branching to a certain type of vascular relation between a leaf and a shoot axillary to it. This relation is illustrated in Fig. 4, B1–B3, p. 299, showing a bract (leaf member) and pedicel (axillary shoot) of *Corydalis nobilis*. When this comparison was suggested for the petal-stamen relation in Hypecoum, it did not appear that the parallel could be carried beyond the stage at which the system, both of axillary shoot and of stamen, consisted of paired bundles facing one another to right and left; for, in the next stage, the pair of bundles for the shoot bud divides to produce a radial system, while the stamen supply, on the other hand, degrades, as a rule, from the level of differentiation which it has reached, and comes to consist of a single bundle, though, in rare instances, it may remain two-bundled. The comparison with an axillary shoot can now, however, be taken a degree farther, for in *Eurycles Cunninghamii* (Fig. 2, G1–G3, p. 296) the stamen bundles, which at first are paired (G2), at a higher level show a tendency to radial arrangement, since one of them has given off an additional small adaxial bundle, with its xylem pointing towards that of the two original strands (G3). In the one flower of which I was able to cut serial section, the extra bundle occurred in four of the six stamens, two in each whorl, but had disappeared in all four before the filament became free from the perianth tube.

This comparison between the vascular relation of a perianth member and its superposed stamen on the one hand, and the vascular relation of a foliage leaf and its axillary branch on the other hand, is an example showing that a common 'Gestalt' basis (Arber, 1937) may underlie two types of structure, though they are not homologous when regarded from the standpoint of formal morphology.

IV. SUMMARY

(i) *The 'corona' in the Amaryllidaceae.*

The form and anatomy of various types of stamen-cup in the Pancratieae (Hymenocallis, &c.) and of the corona in the Coronatae (Narcissus) are compared, and it is concluded that the theory that these structures can be arranged in a series—beginning with the winged stamens of such a form as *Eustephia pamiana*, continuing with the stamen-cup of Hymenocallis, &c., and ending with the free corona of Narcissus—is untenable. The evidence is held to prove that the stamen-cup of the Pancratieae belongs to the androecium alone, while the corona of Narcissus is independent of the stamens. It seems best to interpret it as an outgrowth from the perianth tube, orientated to face the perianth, like the enations of double-bladed foliage leaves.

(ii) *The vascular relation of a perianth member and its superposed stamen.*

The special type of vascular relation existing between a perianth member and a stamen superposed upon it has been described elsewhere for Hypecoum (Arber, 1932), and has been compared with the vascular relation of an axillant leaf to its lateral branch. The Amaryllidaceae furnish examples which confirm this comparison and make it possible to extend it to a further point. This relation is an instance of a single 'Gestalt' type finding its expression in structures which formal morphology would not treat as homologues.

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A Note upon Obione portulacoides (L.) Gaert.

BY

V. J. CHAPMAN, Ph.D.

(*University of Manchester*)

With Plate XIII and two Figures in the Text

DURING the course of an ecological investigation of the salt marshes of Norfolk it was noted that there were at least two distinct forms of *Obione portulacoides*. One is a large bushy form while the other is prostrate. In Moss's Cambridge flora (1914) the large plant is described as *Atriplex portulacoides* var. *latifolia* Guss. and the small one as form *parvifolia* Moss. The descriptions given are very brief and it seemed desirable that they should be amplified. Furthermore, it was hoped that experimental methods would show whether the small plants were only a habitat form or a true variety. In 1910 Rouy had described the small plant as var. *parvifolia*, but Moss evidently considered that it was not worthy of varietal status. The small form occurs on sandy salt marsh near dunes where the nature of the soil may be responsible for its habit, or else cropping by the rabbits (which was known to take place).

EXPERIMENTS

In the summer of 1932 a small area of marsh bearing the prostrate variety was enclosed by wire-netting in order to ensure rabbit-protection. In August 1935 the plants (including new shoots) still retained their characteristic habit. In 1932 two approximately similar small plants of the prostrate variety were also transplanted to an area occupied by the large bushy variety, one of the transplants being enclosed in wire-netting. The soil here had no sand admixed but was of a clay nature. At the same time two of the bushy plants (identical in size) from the creek were transplanted to a site near the dunes, and one of these was also protected by wire-netting. In August 1935 the protected bushy plant had grown considerably, whilst the unprotected plant, although pruned heavily by rabbits each year, still retained its characteristic large leaves and long internodes, even though its stature was but small (Pl. XIII, Fig. 3). Of the two prostrate transplants, the protected plant died in 1934, probably because it had been inadvertently put in a small hollow and had therefore been subject to much standing water and heavy mud deposition on the leaves. The other plant was still living in 1935 and retained its characteristic habit (cf. Pl. XIII, Fig. 2). These experiments suggest that neither change of soil nor cropping by rabbits has any effect on the two forms and that they should therefore be regarded as varieties following Rouy.

[Annals of Botany, N.S., Vol. I, No. 2, April 1937]

TAXONOMY

Moss describes the species and its variety as follows:

Atriplex portulacoides, L. Up to 4 dm. high. Rhizome short, creeping, branched. Stem decumbent, branched, terete below, angular above. Leaves opposite below, opposite or alternate above. Petioles short, 5–10 mm. Laminae of lower leaves elliptical, entire, lobes absent, apex rounded or apiculate. Upper leaves linear, mealy above and below. Inflorescence of terminal and axillary compound spikes. Partial inflorescences interrupted below, a leaf at the base of each. Flowers perfect, or with stamens or carpels functional only. Flowers July to September. Fruiting bracteoles sessile or nearly so, obdeltoid or three-lobed with middle lobe prominent, united two-thirds of way up from the base, either much tubercled, or only slightly so. Seeds small, rugose, compressed.

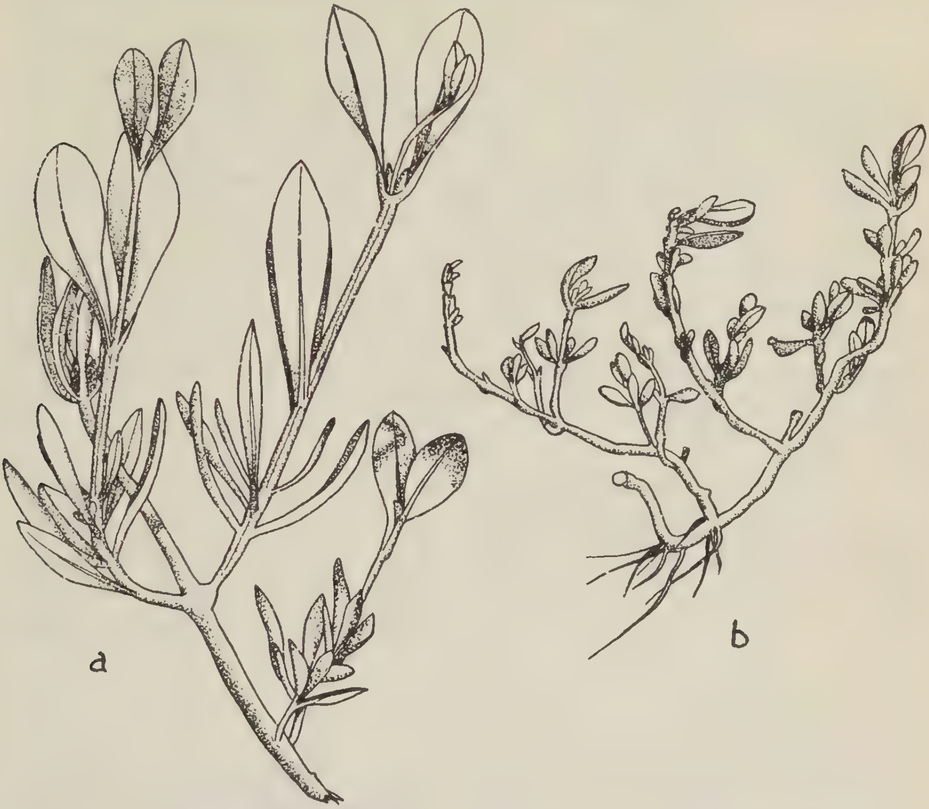
		Var. *latifolia.*	Var. *angustifolia.*	Var. *parvifolia.*
Height (Adults).	Max.	42 cm.	42 cm.	16 cm.
	Min.	30 cm.	30 cm.	7 cm.
Length of internodes.	Max.	5·0 cm.	3·5 cm.	3·1 cm.
	Min.	0·3 cm.	0·3 cm.	0·1 cm.
	Av.	1·6 cm.	1·7 cm.	1·0 cm.
Area of leaf.	Max.	532 sq. mm.	227 sq. mm.	198 sq. mm.
	Min.	293 sq. mm.	120 sq. mm.	101 sq. mm.
Leaves.		3–4 times as long as broad.	6–7 times as long as broad.	3–5 times as long as broad.
		Oblong- or ovato-lanceolate.	Linear-lanceolate.	Oblong- or ovato-lanceolate.
Reproduction.		Flowers, fruits, and seedlings abundant and similar.		Flowers, fruits, and seedlings rare. When present do not differ from the other two vars. Runners and cuttings (Rabbit attack) abundant.

(a) Var. *latifolia*. Laminae oblong-lanceolate, those of main branches three times as long as broad, bracteoles up to 5 mm. long and 4 mm. broad. This is the common British species.

(b) Form *parvifolia*. Dwarf undershrub, 5–6 cm. above ground. Smaller in all its parts. Locally abundant on muddy and sandy salt marshes, rare on shingly marshes.

It seemed desirable, however, that the distinction between the two varieties should be amplified, and so a number of characters were examined in detail in a large number of plants. When this examination was begun it was found that the bushy form appeared in two guises, one having large broad leaves and the other small narrow leaves. Reference to the flora by Moss showed that Gussone (1843) has described a variety *angustifolia* from the Mediterranean. In the herbarium at Manchester University I found plants from Holland

and Belgium which were undoubtedly var. *angustifolia* since they agreed with specimens from the south of France and Corsica. Among the British sheets there were specimens from Sussex, Fleetwood, and Hunstanton which appeared to approach nearly to this variety.

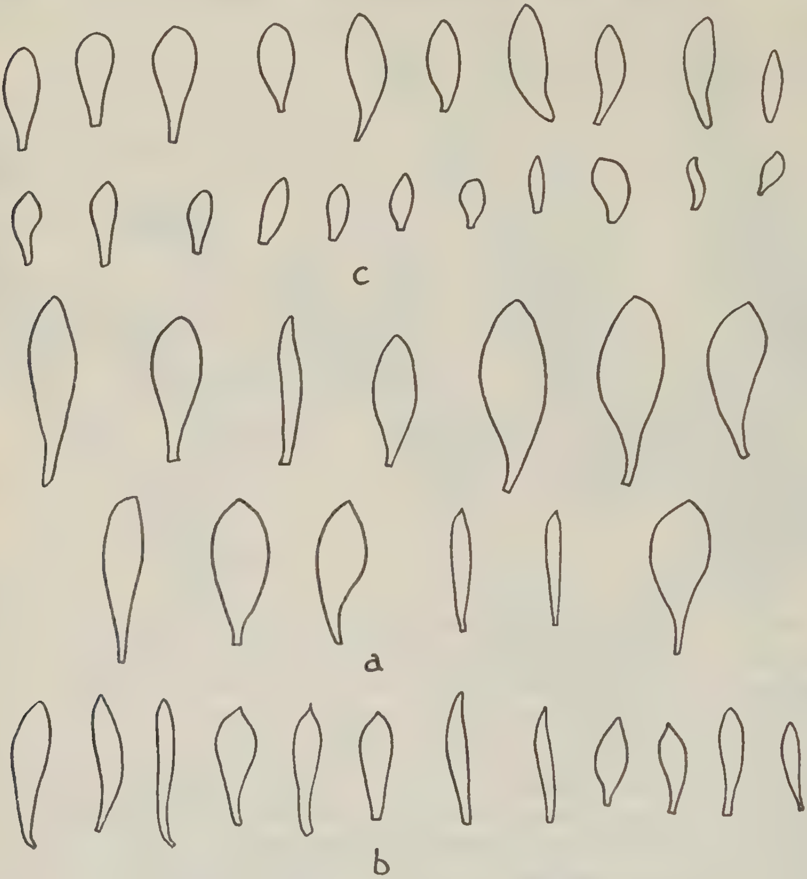


TEXT-FIG. 1. (*a*) *Obione portulacoides* var. *latifolia*—shoot ($\times \frac{3}{4}$). (*b*) *Obione portulacoides* var. *parvifolia*—part of plant ($\times \frac{5}{4}$).

The differences between the varieties are listed in detail on p. 306, and some are shown in Text-figs. 1 and 2.

It will be seen that the three varieties differ principally in leaf size and shape, and in the length of the internodes, var. *parvifolia* being distinguished by its dwarf nature. Vars. *latifolia* and *angustifolia* can hardly be regarded as growth forms since they occur side by side under the same conditions. It is not impossible that there may be intermediates between var. *latifolia* and var. *angustifolia*. These are most likely to occur on the Continent, especially in France, if they are to be found at all. A genetical study of these two varieties is probably desirable.

Apart from the morphological differences described above there is also a marked difference in distribution. In Norfolk both the large varieties fringe the creeks of marshes (Pl. XIII, Fig. 1), and also occur along the edges of shingle ridges. An ecological investigation has strongly suggested that the distribution of var. *latifolia* may be controlled by the drainage conditions. Submergence by the tide or periods of exposure cannot play a great part in determining its distribution because the plants occur over a very wide range of level. Var. *parvifolia*, on the other hand, is confined at Scolt, in Norfolk, to a relatively small range on the marshes, +8·19—10·11 ft. O.D. as compared to +6·66—10·17 ft. O.D. for var. *latifolia*.[1] In the case of var. *parvifolia*, therefore, it is possible that the degree of submergence, since the plant occurs at high levels, may play some part in determining its distribution.



TEXT-FIG. 2. Typical leaves of the varieties of *Obione portulacoides*. (*a*) var. *latifolia*—large-leaved form. Max. area 532, av. 293 sq. mm. (*b*) var. *angustifolia*—small-leaved form. Max. area 227, av. 120 sq. mm. (*c*) var. *parvifolia*. Max. area 198, av. 101 sq. mm. (all × ½).

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[1] There are plants of this variety on the mainland ridges growing at levels that must be considerably higher.

At the same time, however, observations show that this variety usually occurs near dunes where the soil is sandy, and soil composition may also play some part in determining its presence. The different habitats of these two varieties is one of the features that serves to distinguish them.

Amplified descriptions of the three varieties are appended.

Obione portulacoides (L.) Gaert. var. *latifolia* (Gussone) comb. nov.

Laminae oblong- or ovato-lanceolate, those of main branches three to four times as long as broad, bracteoles up to 5 mm. long and 4 mm. broad. Plants up to 4 dm. high, average about 3·5 dm. Flowers, fruits, and seedlings abundant. Salt marshes, often fringing creeks, and shingle ridges. Vertical range large. Frequent.

Obione portulacoides (L.) Gaert. var. *parvifolia* Rouy.

Dwarf undershrub, 5–16 cm. high, prostrate, straggling. Smaller in all its parts. Leaves oblong- or ovato-lanceolate. Flowers, fruits, and seedlings rare. Reproduction largely by vegetative means. Locally abundant on sandy salt marshes and in lows in the dunes. Vertical range narrow. At present from Norfolk and Brittany.

Obione portulacoides (L.) Gaert. var. *angustifolia* (Gussone) comb. nov.

Laminae linear-lanceolate, those of main branches six to seven times as long as broad. Plants up to 4 dm. high. Flowers, fruits, and seedlings abundant. Salt marshes and shingle beaches. Norfolk, Lancashire, Sussex, Belgium, Holland, France, Corsica.

SUMMARY

1. Experiments upon two forms of *Obione portulacoides* are described, and it is suggested that they are true varieties.

2. The differences between the two varieties are described in detail, together with a third variety from France.

3. The previous short descriptions of the varieties are amplified by detailed descriptions.

The author gratefully acknowledges the help and advice of Dr. H. Godwin during the course of the investigation.

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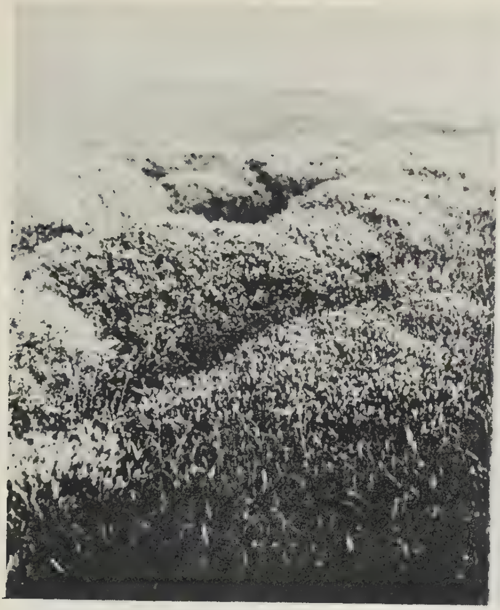
EXPLANATION OF PLATE XIII

Illustrating Dr. V. J. Chapman's paper on 'A Note upon *Obione portulacoides* (L.) Gaert.'

Fig. 1. *Obione portulacoides* var. *latifolia* fringing a creek.

Fig. 2. Transplant of *O. portulacoides* var. *parvifolia* amongst var. *latifolia*. After two years. The plant is indicated by the arrow, and its small leaves show up against the case in contrast with the larger leaves of the surrounding plants of the other variety.

Fig. 3. Transplant of *O. portulacoides* var. *latifolia* near dunes. After two years. The plant on the left has been cropped by rabbits but still maintains its large leaves. Plants of var. *parvifolia* can be seen to the right of the netting and behind the cropped plant.



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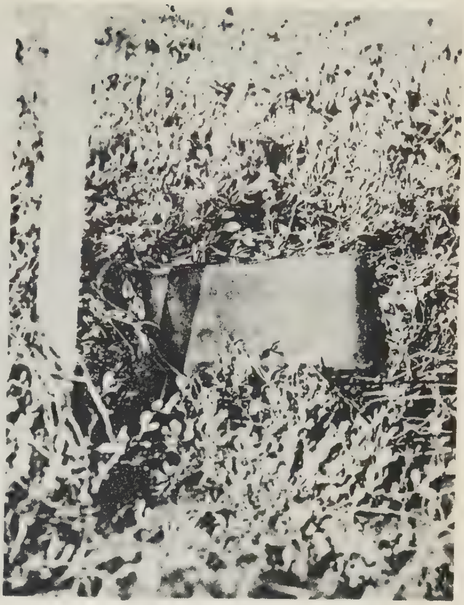

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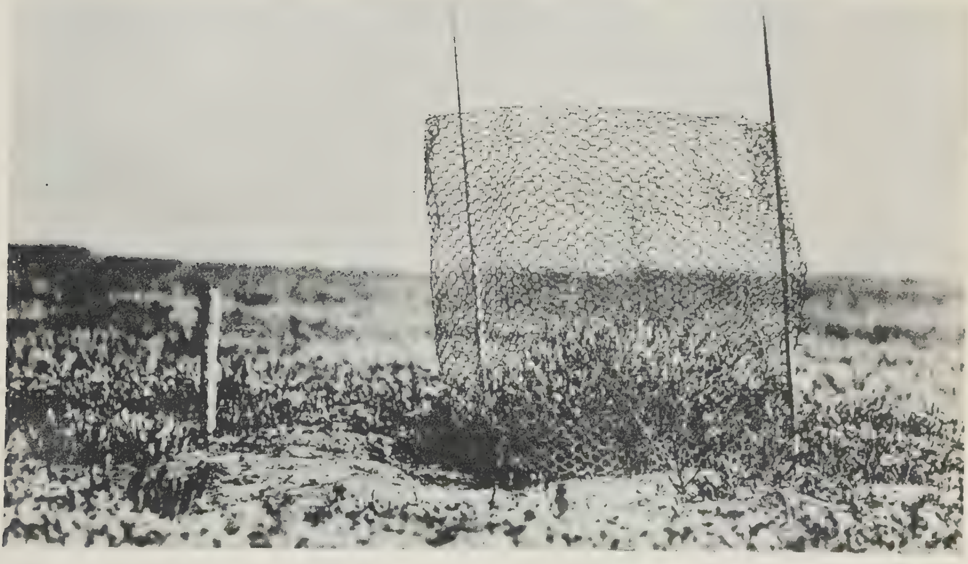

3

CHAPMAN — OBIONE.

Huth.Stubbs X. Kent

The Response of the Respiratory System in Mango and Guava to Alteration in the Concentrations of Oxygen and Nitrogen

BY

B. N. SINGH, D.Sc.

(*Kapurthala Professor of Agricultural Botany and Plant Physiology and Director of the Institute of Agricultural Research, Benares Hindu University, India*)

P. V. V. SESHAGIRI, M.Sc.

AND

S. S. GUPTA, M.Sc.

With four Figures in the Text

INTRODUCTION

A STUDY of the relationship between aerobic and anaerobic respiration has been engaging the attention of plant physiologists for a long time. Blackman and Parija (1928) studied the respiration of a population of senescent ripening apples, and Parija (1928) investigated the relationship between respiration of apples in nitrogen and in air. From an analytical study of these researches F. F. Blackman (1928) has elaborated a scheme to explain the respiratory process in apples and plants in general. Gustafson (1930), working on intramolecular respiration of tomato fruits, observed an increase in carbon dioxide production on depriving fruits of oxygen by substituting nitrogen or hydrogen. Investigations into the subject of relative effects of air and nitrogen on certain fat-storing and carbohydrate-storing seeds have been described by Leach and Dent (1934) and Leach (1936), from which it is concluded that the respiratory processes of the actively growing tissues of germinating seeds differ in important details from those observed by Blackman in ripening apples.

Detailed investigations have been in progress in these laboratories for the last few years on the respiration of various tropical plants and plant organs under different intensities of external and internal factors such as temperature, oxygen, nitrogen, starvation, age, season, injection of solutions, &c.; from analytical studies of these a schema of the mechanism of respiration was formulated in 1926 (Singh, 1930). Investigation has been continued since then to throw further light on the mechanism of respiration.

Along with a study of the various methods of storing and preserving fruits

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it is considered important to find out the natural drift of their respiration when taken from storage and allowed to respire at ordinary temperatures, and to study the effects of changes of environment from air to nitrogen and vice versa. Further a study of the response of the respiratory system to varying atmospheres may give a clue to the nature of the substrate for respiration, and also as to the critical percentage of oxygen likely to be helpful in prolonging the storage life of the fruits.

In the present paper are described the results of investigations of the respiration of mango and guava in air, in nitrogen, and in oxygen and nitrogen of various concentrations.

Experimental Methods and Procedure

Mature mangoes and guavas were kept in respiration chambers fully immersed in a constant temperature bath thermostatically maintained at a temperature of 30° C. The three-hourly output of CO_2 was measured by absorption in standard baryta solution in Pettenkoffer tubes; by using the Blackman's commutator continuous records of the production of CO_2 were obtained for a period of four to eight days.

Mangoes brought from cold store (8° C.) were used in the respiration chamber. Their course of respiration in air was first studied; the air stream after some time was replaced by a stream of nitrogen from a cylinder after passing through alkaline pyrogallol solution to remove traces of oxygen. The effect of a change back to air was also studied.

The study was further extended to guavas gathered from the orchard. Guavas were allowed to respire in an atmosphere of nitrogen for a period of forty-five hours and the response to a change to air was then studied; later there was a transference to nitrogen after fifteen hours' respiration in air.

Different mixtures of oxygen and nitrogen were prepared in large containers by mixing accurate amounts of gases obtained from cylinders of oxygen and nitrogen. The mixtures thus obtained were analysed by Singh and Mathur's (1937) modified Haldane's gas analysis apparatus. Mature mangoes were kept in respiration chambers and their CO_2 output and oxygen intake in air were measured by the above apparatus. The air was then replaced by the particular gas mixture and the gaseous exchange measured after an hour. The gas mixture was again replaced by air and the gaseous exchange further studied.

Experimental Findings

Course of respiration of mangoes and guavas under laboratory conditions.

The mangoes begin with an initial high value of carbon dioxide production which declines with time, showing slight fluctuations (Fig. 1 (*a*) and Table I, Appendix). For the first thirty hours the course of respiration is fairly steady followed by a gradual fall. It again assumes a steady level phase for about thirty-five hours (60–95), declining continuously till the end of the experiment. A similar course of respiration is observed in guavas (Fig. 1 (*b*)).

The presence of some respirable substrate in one form or other is essential for respiration. As the fruits are isolated from the tree the respirable substrate present becomes slowly depleted, and this is concomitant with the steady phase observed. With advance of time the available respirable substrate is much reduced and the intensity of respiration declines. In isolated plant organs like fruits there are two opposite forces conditioning the production of carbon dioxide. One is that of senescence which tends to bring about a lowering of the 'organizational resistance' of the tissue, resulting in a faster rate of hydrolysis of the reserve substances leading to a higher rate of respiration, as was observed in a previous communication from this laboratory (Singh, Seshagiri, and Gupta, 1937); the other is that of natural starvation which tends to counteract the action of the former. The respiration thus observed is possibly a resultant of the two opposite forces.

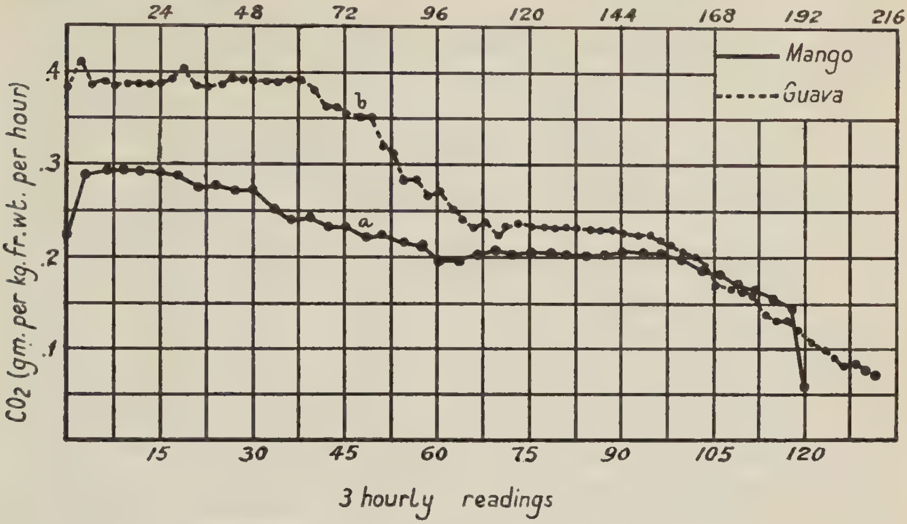

FIG. 1. Course of respiration of Langra mangoes (*a*) and guavas (*b*) under laboratory conditions.

The initial effect of change of temperature and 'air-nitrogen-air' effects.

Mangoes respiring at a rate of 0·125 gm. per kg. fresh weight per hour when in cold store (8° C.) show a high rate of respiration (0·450) as soon as they are brought to the laboratory temperature of 30° C. (Fig. 2 (*a*) and Table III, Appendix). The enhanced carbon dioxide output may be attributed to an alteration in the equilibrium of its solution in the water of the intercellular spaces, caused by a higher temperature.

The magnitude of respiration then declines rapidly for the first twenty hours and maintains a level phase for the next fifteen. At low temperatures the sugars steadily accumulate at the expense of starch, a fact to be described in the next paper of the series. When the fruits are brought to the high temperature of the laboratory from the cold store the accumulated sugars are possibly reconverted to starch to maintain the starch-sugar equilibrium.

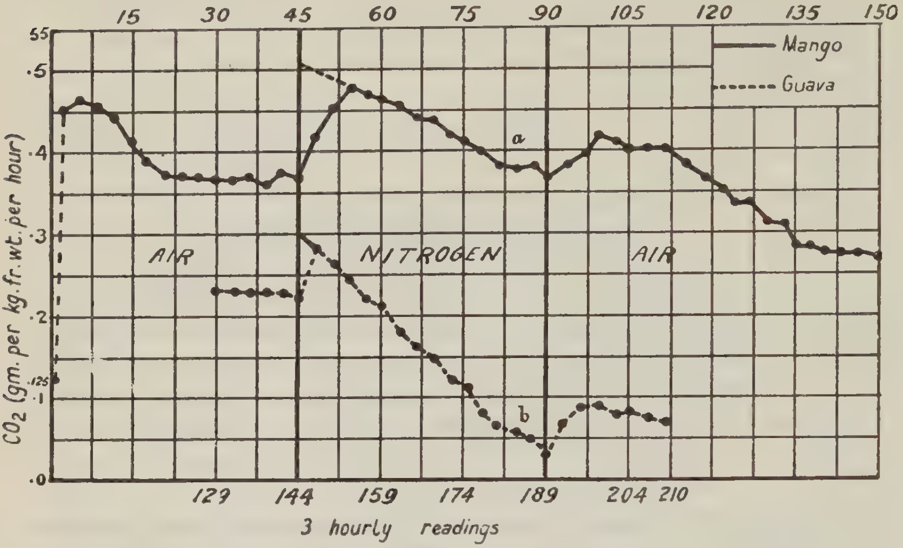

FIG. 2. 'Air-nitrogen-air' effects on the respiratory behaviour of Langra mangoes (*a*) and guavas (*b*).

With a sudden change from low to a high temperature the high concentration of sugars present and forming the substrate for respiration leads to an enhanced rate of carbon dioxide output. With the reconversion of sugars to starch, perhaps to attain an equilibrium at the higher temperature, there is a drop in the intensity of respiration. Once the equilibrium is restored the course of respiration shows a level phase.

An initial peak in respiratory intensity is absent in the guavas (Table II, Appendix); they are brought for experimentation from the orchard and kept in the laboratory for a few hours before they are studied, while mangoes are brought from cold store.

After a steady phase of respiration has been attained the air current is replaced by pure nitrogen in both the fruits (Fig. 2). A sudden rise in carbon dioxide output is observed, followed by a sharp decline approaching a level phase in the end. After forty-five hours' respiration in nitrogen the gas is again replaced by an air stream. This change is also accompanied by an increase in respiration followed by a steady decline till the end.

On replacement of air by nitrogen the carbon dioxide output rises. At the moment of the change, the substrate for nitrogen respiration may have been the product of glycolysis in air. If the declining series of values in the graphs are extrapolated backwards to the zero line of transference to nitrogen a measure of the glycolysis in air previous to the treatment of nitrogen is obtained. On analysing the data after the method of Blackman (1928) it is found that the value of nitrogen respiration is 1·36 and 1·34 times that of air in the case of guava and mango respectively. The substrate for respiration at the time when nitrogen is introduced may be expected to be a result of the glycolysis in air. The products of nitrogen respiration are alcohol and carbon dioxide.

$$C_6H_{12}O_6 = 2C_2H_5OH+2CO_2.$$

So that the value of glycolysis is three times that of nitrogen respiration (6C/2C = 3)

$$\begin{aligned} NR/OR &= 1{\cdot}34 \text{ (or } 1{\cdot}36) \quad \therefore\ NR = 1{\cdot}34\ OR \text{ (or } 1{\cdot}36\ OR) \\ GL &= 3\ NR \quad \therefore\ GL = 3 \times 1{\cdot}34\ OR \text{ (or } 3 \times 1{\cdot}36\ OR) \\ &\qquad\qquad = 4{\cdot}02\ OR \text{ (or } 4{\cdot}08\ OR), \end{aligned}$$

where NR, OR, and GL stand for nitrogen respiration, air respiration, and glycolysis respectively.

From the equations so deduced it appears that out of every four atoms of carbon present at the glycolytic stage only one appears as carbon dioxide in air respiration, and there is no evidence of the formation of a secondary product like alcohol such as is found in nitrogen respiration; the remaining three atoms of carbon are possibly built back into the system into some stage previous to glycolysis. This process is termed 'synthetic oxidation' (Singh, 1930), as suggested in the researches on respiration of oranges and Knolkhol tubers in these laboratories, or 'oxidative anabolism', as Blackman (1928) termed it. Leach (1936), however, did not obtain any evidence of the occurrence of oxidative anabolism in the respiration of germinating seeds, and the glycolysis values obtained were very low. It is of interest to note that the values of glycolysis obtained in the senescent ripening mangoes and guavas in this work and those obtained by Blackman in his studies on senescent ripening apples agree fairly closely; the slight differences may be taken to be due to differences in material. But the very low measure of glycolysis obtained in germinating seeds are explained by Leach as due to important differences in the respiratory processes of the actively growing tissues of germinating seeds and the senescent tissues of ripening fruits.

The continued absence of oxygen has, it seems, limited the substrate for glycolysis, resulting in a reduced rate of respiration. The final change from nitrogen to air supplies the oxygen necessary for the preglycolytic stages, thus leading to the production of the substrate for glycolysis. With increase in the substrate for glycolysis there is an enhancement in carbon dioxide output. The fall in the respiratory intensity during the last stages of the experiment is evidently due to the exhaustion of reserve food materials and the occurrence of starvation.

The 'nitrogen-air-nitrogen' after-effects.[1]

The intensity of respiration of guavas is very high when the fruits are first placed in nitrogen (Fig. 3). The carbon dioxide output rapidly declines, attaining a level phase in a short time. When the nitrogen stream is switched off and air admitted (144 hours) there is a rapid rise in the intensity of respiration for about thirty hours, becoming steady later. After attaining a steady phase the air is replaced by nitrogen, when the respiratory rate decreases rapidly after a slight rise.

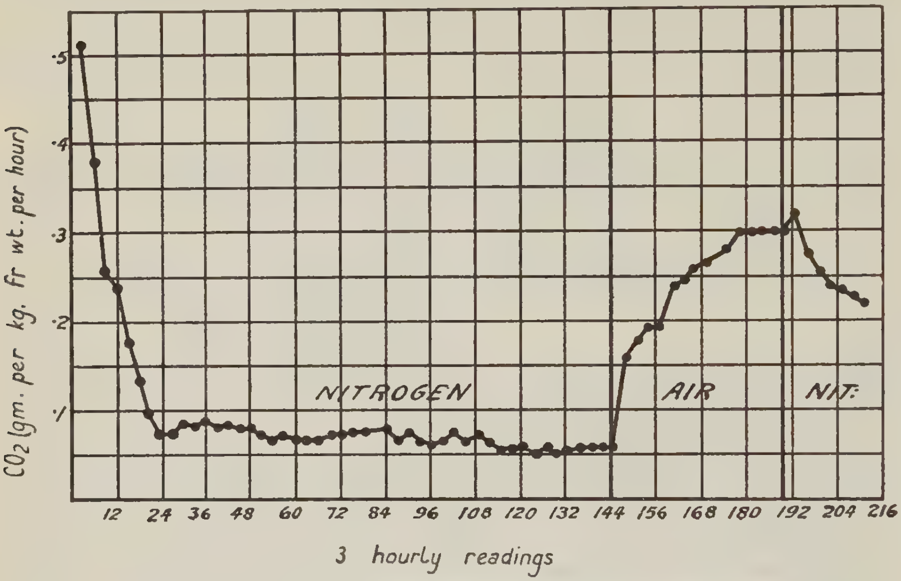

FIG. 3. 'Nitrogen-air-nitrogen' after-effects on the respiratory behaviour of guavas.

The initial high respiratory values may be due to a change from the aerobic to anaerobic conditions. On comparing the values of respiration in air with those of nitrogen, the latter are about 1·25 times the former. Glycolysis value in air according to this ratio works out at 3·75 OR, approaching quite closely that for the guavas previously investigated.

It has been previously observed that the greater output of carbon dioxide when the fruits are changed from nitrogen to air may be the effect of oxygen on the preglycolytic reactions, resulting in a greater production of substrate for glycolysis. On the basis of the present data another explanation is possible. In nitrogen respiration it may be that a compound or compounds are formed which, when the fruits are brought back to oxygen, are oxidized to carbon dioxide and water. It would thus appear that shorter or longer periods of nitrogen treatments should produce smaller or greater amounts of such compounds, which in their turn, on exposure to air, produce carbon dioxide in the same proportions. A study of the results obtained by subjecting the guavas to anaerobic conditions for shorter (45 hours) and longer periods (145 hours) shows that the amounts of carbon dioxide output, or the magnitudes of the respiratory humps (Figs. 2 and 3), when the fruits are changed from nitrogen to air, are directly proportional to the durations of the nitrogen treatment.

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[1] Records on this aspect of the investigation were obtained on the guava fruit alone.

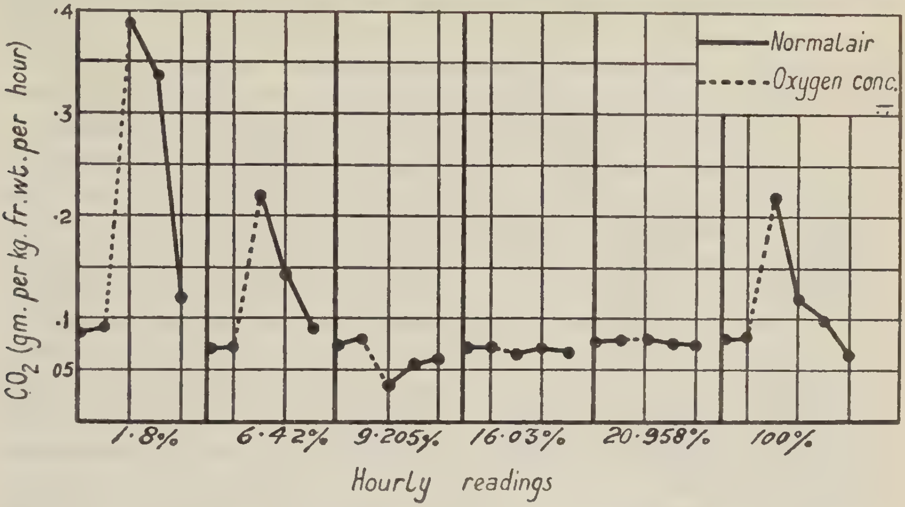

FIG. 4. Respiratory behaviour of mangoes in varying concentrations of oxygen in nitrogen.

Respiratory behaviour of fruits in varying concentrations of oxygen and nitrogen.

Mature mangoes are allowed to respire in air and after measuring the respiratory drift are treated with the particular gas mixture. After taking a few hourly records the gas mixture is replaced by air and the respiratory drift determined again.

The output of carbon dioxide in normal air rises and falls slightly. The introduction of 1·8 per cent. oxygen with nitrogen, however, enhances the rate of carbon dioxide production (Fig. 4). The oxygen concentration present in the mixture is probably insufficient for full aerobic respiration, as a result of which anaerobiosis predominates and the amount of carbon dioxide production increases. The use of 6·42 per cent. oxygen mixture in place of air increases the carbon dioxide output but the rate is less than in 1·8 per cent. oxygen. The analysis of the gas shows that there is only a small intake of oxygen (Table V), suggesting the possibility of oxygen and nitrogen respiration occurring together.

Fruits respiring in a gas mixture of 9·2 per cent. oxygen exhibit a minimum respiratory rate, i.e. one which is lower than that in any gas mixtures of higher or lower oxygen content. It may be that oxygen present in such concentration is just sufficient to extinguish the process of anaerobic respiration. In the lower concentrations of oxygen there is probably a combination of aerobic and anaerobic respiration, the latter predominating in the lower concentrations. In a gas mixture of 9·2 per cent. oxygen it is possible that a point is reached when just enough oxygen enters the cells to stop anaerobic respiration and to carry on the pre- and post-glycolytic reactions. If the oxygen intake in this critical concentration of the mixture is just sufficient to effect complete oxidation of the products of glycolysis present, the respiratory quotient is likely to throw some light on the nature of the substrate. It is found that the value of the respiratory quotient at this stage is 0·85 (Table V, Appendix).

If carbohydrate alone be the nature of the substrate for respiration the expected values of the quotient should be unity. If the principal substrate for respiration is of the nature of fat (such as $C_{57}H_{104}O_9$), and if that alone is broken down in respiration, the respiratory quotient works out at about 0·73. The respiratory quotient should naturally vary with the nature of the substrate. A substrate of a mixture of fat and carbohydrate, when respired in equal proportions, gives a different value for the respiratory quotient, $1{\cdot}0+0{\cdot}726/2 = 0{\cdot}86$. The respiratory quotient of the mangoes at the critical concentration of oxygen (9·205 per cent.) works out to be 0·85. It may be inferred from this that the nature of the substrate in mangoes is a mixture of fats and carbohydrates.

The carbon dioxide output in 16·03 per cent. oxygen is lesser than in any of the lower concentrations (Fig. 4), yet higher than that in 9·2 per cent. oxygen. There is still a depression in carbon dioxide production on transference from air to the mixture. The oxygen intake too is more than that in the former cases (Table V, Appendix), showing this to be a result of complete aerobic respiration. When mangoes are given 100 per cent. oxygen there is an enormous increase in the output of carbon dioxide due possibly to an acceleration of pre- and post-glycolytic reactions. On replacement of oxygen by air a high rate is exhibited for some time which later drops. The high concentration of the products of glycolysis formed in 100 per cent. oxygen keep up the rate even on removing the fruits to an atmosphere of air (21 per cent. O_2). The oxygen intake is highest in 100 per cent. oxygen, yet the quotient is greater than unity (Table V, Appendix).

A review of the effects of these varying oxygen mixtures on the respiratory behaviour of the mango indicates that the carbon dioxide output is highest at the lowest concentration of oxygen (1·08 per cent.). With an increase in the percentage of oxygen, the respiratory intensity appreciably falls until a minimum is reached at 9·2 per cent. A further rise in oxygen concentration enhances the rate of respiration and the maximum carbon dioxide production is observed with 100 per cent. oxygen. An oxygen mixture of 9·2 per cent. thus appears to be the critical concentration, increase or decrease beyond which results in a higher rate of carbon dioxide production. A high rate of respiration leads to a rapid depletion of the reserve food material and to a rapid completion of the senescent phase of isolated plant organs, such as fruits; as reported in a previous communication (Singh, Seshagiri, and Gupta, 1937). A low rate of respiration depletes the food materials very slowly and the senescent phase of the fruit is prolonged. On the basis of the above observations it may be suggested that a gas mixture of 9·2 per cent. oxygen be tried as an atmosphere in which mangoes can be advantageously preserved.

SUMMARY AND CONCLUSIONS

The respiratory behaviour of mangoes and guavas under varying gaseous environments are studied at a temperature of 30° C.

The course of respiration of the mature mango and guava begins with a high value, becoming steady later and falling off in the end owing to starvation. An enhancement of carbon dioxide output is observed on transferring mangoes from cold store at 8° C. to 30° C. An alteration in the equilibrium of solution of carbon dioxide in water present in the intercellular spaces due to the change of temperature may be the cause of this.

Mangoes brought from air to an atmosphere of nitrogen exhibit an increased carbon dioxide output and a change from nitrogen to air also enhances the rate of carbon dioxide production. The value of glycolysis worked out on the Blackman (1928) assumptions is found to be four times that of oxygen respiration, while three-fourths of the carbon taking part in glycolysis is possibly built back into the system.

The intensity in the rate of carbon dioxide production on changing from nitrogen to air is directly proportional to the length of the period of anaerobiosis to which the fruit is subjected.

The carbon dioxide output is at a minimum in 9·2 per cent. oxygen, an increase or a decrease of this tends to increase the carbon dioxide production. An oxygen mixture of 9·2 per cent. is the critical concentration below which there is probably a competition between aerobiosis and anaerobiosis. The respiratory quotient at this critical point is 0·85, suggesting the possibility of a mixture of fats and carbohydrates being the substrate for respiration.

It is suggested that 9·2 per cent. oxygen may be used as an atmosphere in which mangoes can be stored with a view to prolonging the senescent ripening period.

APPENDIX

TABLE I

Course of Respiration of Mature Mangoes (Langra) at 30° C.

CO_2 expressed as gm. per kg. fresh wt. per 3 hours.

Hours.	CO_2.	Hours.	CO_2.
3	0·292	63	0·198
6	0·292	66	0·200
9	0·293	69	0·205
12	0·292	72	0·201
15	0·290	75	0·202
18	0·285	78	0·203
21	0·275	81	0·202
24	0·275	84	0·202
27	0·270	87	0·208
30	0·271	90	0·206
33	0·250	93	0·203
36	0·239	96	0·202
39	0·239	99	0·198
42	0·230	102	0·186
45	0·230	105	0·181
48	0·221	108	0·170
51	0·221	111	0·164
54	0·216	114	0·155
57	0·210	117	0·147
60	0·198	120	0·051

TABLE II

Course of Respiration of Mature Guavas at 30° C.

CO_2 expressed as gm. per kg. fresh wt. per 3 hours.

Hours.	CO_2.	Hours.	CO_2.
3	0·410	63	0·381
6	0·385	66	0·361
9	0·388	69	0·360
12	0·383	72	0·353
15	0·385	75	0·351
18	0·385	78	0·350
21	0·382	81	0·321
24	0·385	84	0·310
27	0·389	87	0·281
30	0·400	90	0·288
33	0·383	93	0·266
36	0·380	96	0·270
39	0·385	99	0·251
42	0·392	102	0·240
45	0·390	105	0·230
48	0·391	108	0·235
51	0·387	111	0·220
54	0·388	114	0·230
57	0·390	117	0·235
60	0·389	120	0·231

TABLE II (*contd.*)

Hours.	CO_2.	Hours.	CO_2.
123	0·230	168	0·169
126	0·229	171	0·165
129	0·230	174	0·161
132	0·228	177	0·160
135	0·228	180	0·136
138	0·228	183	0·131
141	0·227	186	0·130
144	0·221	189	0·120
147	0·221	192	0·108
150	0·220	195	0·100
153	0·215	198	0·092
156	0·210	201	0·081
159	0·203	204	0·083
162	0·201	207	0·075
165	0·190	210	0·070

TABLE III

'Air-nitrogen-air' Effects on Mangoes (Langra) Removed from Cold Store to 30° C.

CO_2 expressed as gm. per kg. fresh wt. per 3 hours.

Hours.	CO_2.	Hours.	CO_2.
0 (Air)	0·115	78	0·400
3	0·450	81	0·385
6	0·460	84	0·380
9	0·455	87	0·385
12	0·445	90	0·372
15	0·410	93	0·382
18	0·381	Changed to air	
21	0·375	96	0·395
24	0·372	99	0·415
27	0·370	102	0·410
30	0·370	105	0·405
33	0·369	108	0·402
36	0·370	111	0·400
39	0·360	114	0·385
42	0·378	117	0·372
45	0·372	120	0·361
48	0·418	123	0·342
Changed to nitrogen		126	0·345
51	0·452	129	0·324
54	0·480	132	0·318
57	0·470	135	0·292
60	0·462	138	0·291
63	0·455	141	0·285
66	0·440	144	0·284
69	0·442	147	0·282
72	0·421	150	0·275
75	0·410	—	—

TABLE IV

Air-nitrogen-air and Nitrogen-air-nitrogen Effects on Guavas at 30° C.

CO_2 expressed as gm. per kg. fresh wt. per 3 hours.

Hours.	CO_2. Air	CO_2. Nitrogen	Hours.	CO_2. Air	CO_2. Nitrogen
3	0·410	0·509	108	0·235	0·072
6	0·385	0·375	111	0·220	0·062
9	0·388	0·252	114	0·230	0·058
12	0·383	0·237	117	0·235	0·059
15	0·385	0·175	120	0·231	0·060
18	0·385	0·133	123	0·230	0·053
21	0·382	0·092	126	0·229	0·057
24	0·385	0·074	129	0·230	0·052
27	0·389	0·073	132	0·228	0·052
30	0·400	0·085	135	0·228	0·056
33	0·383	0·083	138	0·228	0·056
36	0·383	0·087	141	0·227	0·058
39	0·385	0·080	144	0·221	0·058
				Nitrogen	Air
42	0·392	0·083	147	0·281	0·057
45	0·390	0·078	150	0·268	0·178
48	0·391	0·079	153	0·245	0·190
51	0·387	0·073	156	0·220	0·191
54	0·388	0·068	159	0·212	0·191
57	0·390	0·070	162	0·108	0·243
60	0·389	0·067	165	0·162	0·255
63	0·381	0·065	168	0·150	0·262
66	0·361	0·064	171	0·122	0·261
69	0·360	0·070	174	0·114	0·275
72	0·353	0·073	177	0·082	0·292
75	0·351	0·074	180	0·065	0·294
78	0·350	0·075	183	0·060	0·297
81	0·321	0·074	186	0·051	0·298
84	0·310	0·077	189	0·031	0·299
				Air	Nitrogen
87	0·281	0·066	192	0·072	0·315
90	0·288	0·075	195	0·090	0·270
93	0·266	0·065	198	0·092	0·251
96	0·270	0·062	201	0·081	0·238
99	0·251	0·066	204	0·083	0·239
102	0·240	0·075	207	0·075	0·225
105	0·230	0·068	210	0·070	0·217

TABLE V

Respiration of Mature Mangoes (Langra) in Various Concentrations of Oxygen at 30° C.

CO_2 and O_2 expressed as gm. per kg. fresh wt. per hour.

Respiration in air.			Respiration in oxygen.				Respiration in air again.		
CO_2.	O_2 intake.	$\frac{CO_2}{O_2}$	O_2 (%).	CO_2.	O_2 intake.	$\frac{CO_2}{O_2}$	CO_2.	O_2 intake.	$\frac{CO_2}{O_2}$
(1) 0·085	0·093	0·91	1·8	(3) 0·385	nil	—	(4) 0·335	0·515	0·65
(2) 0·091	0·100	0·91					(5) 0·120	0·154	0·78
(1) 0·070	0·082	0·85	6·42	(3) 0·220	0·049	4·49	(4) 0·145	0·193	0·75
(2) 0·072	0·089	0·81					(5) 0·090	0·109	0·82
(1) 0·075	0·083	0·89	9·20	(3) 0·035	0·041	0·85	(4) 0·055	0·070	0·79
(2) 0·080	0·092	0·87					(5) 0·060	0·076	0·79
(1) 0·070	0·074	0·95	16·03	(3) 0·065	0·071	0·91	(4) 0·070	0·081	0·86
(2) 0·070	0·073	0·96					(5) 0·068	0·077	0·88
(1) 0·075	0·090	0·83	20·95	(3) 0·080	0·099	0·81	(4) 0·075	0·093	0·81
(2) 0·078	0·093	0·84					(5) 0·070	0·085	0·82
(1) 0·080	0·094	0·85	100·00	(3) 0·220	0·190	1·15	(4) 0·120	0·077	1·55
(2) 0·085	0·104	0·82					(5) 0·098	0·070	1·40
							(6) 0·065	0·046	1·40

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Leaf Arrangement in Relation to Water Conduction in the Foliose Hepaticae

BY

DAVID A. CLEE

(Department of Biology, University College of Swansea)

With two Figures in the Text

DURING an investigation into the mechanism of water conduction in the foliose Hepaticae the writer was struck by the relative prevalence of succubous over incubous forms, and decided to determine whether the leaf arrangement bore any relation to the efficiency of water conduction.

Plagiochila asplenioides var. *major* (Nees), found growing on damp, shaded banks, completely covered by the surrounding vegetation of moss and grass, was chosen for experimental purposes on account of its length (6–10 cm.), succubous arrangement, erect habit, and the absence of amphigastria.

The cut ends of several plants were blocked by leaving them dipping in melted paraffin wax for two minutes and then hardening the wax by immersing in cold water for five minutes. After the surplus water had been removed by means of blotting-paper the plants were placed in small glass stands constructed in the following way. Pieces of glass tubing 2 cm. long and 0·7 cm. in diameter were cemented to small glass plates at an average distance of 1·0 cm. apart. The tubes were filled with a 0·5 per cent. solution of eosin, and a single plant was inserted into each to a depth of 0·5 cm. A little glass wool was used in the mouth of the tube to maintain each plant in an erect position, and the distance of the tubes apart ensured the isolation of the individual plants. The prepared stands were then placed in an enclosed glass chamber containing a little water, in order to ensure the moist atmosphere normal to the plants (Fig. 1).

By the aid of the reading microscope the rise of the eosin solution could easily be detected by the steady accumulation of the stain in the leaf axils. Readings were taken at intervals with the following typical results:

TABLE I

Plant No.	Length of plant in cm.	External rise of eosin solution in cm. in 1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	24 hrs.
1	4·5	0·25	0·51	0·89	1·07	1·5	1·9	2·25
2	5·1	0·48	0·54	0·7	0·9	1·07	1·15	1·69
3	7·8	0·9	0·98	1·2	1·6	1·87	2·04	2·38
4	3·8	0·34	0·53	0·58	0·69	0·78	0·97	1·55
5	4·5	0·53	0·71	0·81	0·99	1·26	1·31	1·68

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Exactly similar experiments were then carried out with inverted plants, the tips instead of the cut ends being inserted approximately 0·5 cm. into the tubes. The inversion converted the succubous into an incubous arrangement, as viewed from below upwards, and typical readings of the rate of ascent of the eosin under these conditions are shown below:

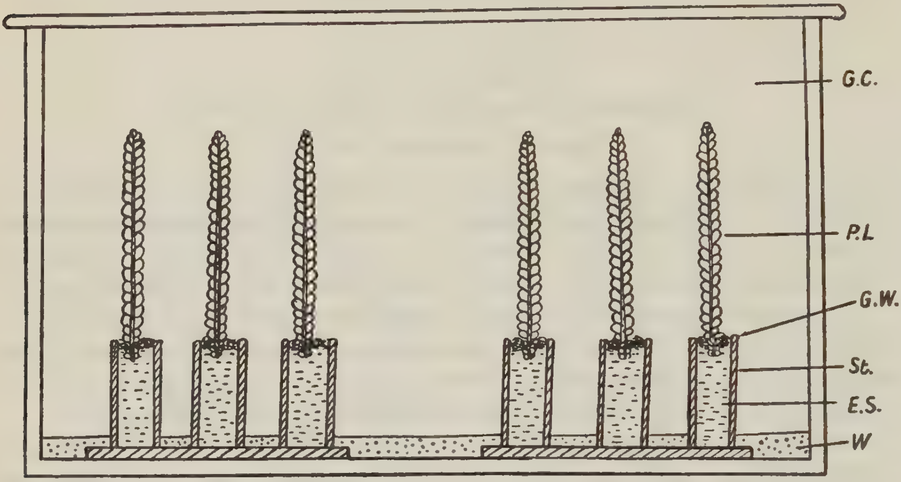

FIG. 1. Diagram of apparatus used, G.C., glass chamber; PL., plant; ST., stand; G.W., glass wool; E.S., eosin solution; W., water.

TABLE II

Plant No.	Length of plant in cm.	External rise of eosin in cm. in 1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	24 hrs.
1	3·5	0·1	0·15	0·3	0·38	0·45	0·5	0·53
2	4·2	0·16	0·24	0·33	0·59	0·6	0·8	0·84
3	3·7	0·2	0·51	0·6	0·65	0·67	0·68	0·71
4	4·6	0·6	0·66	0·67	0·7	0·81	0·9	0·94
5	4·0	0·3	0·4	0·43	0·55	0·6	0·62	0·93

From a comparison of these figures it is clear that in *Plagiochila asplenioides* var. *major* the rate of ascent of water from the substratum over the external surface is decidedly more rapid when the leaf arrangement is in the succubous than when it is in the incubous condition.

An attempt was then made to determine the rates of transport of a water film from the centre to the tip and base respectively of the same plant, the portion from the centre to the tip corresponding to a succubous, and that from the centre to the base, to an incubous, arrangement in relation to the situation of the water supply. A siphon consisting of a finely drawn-out glass tube containing a 0·5 per cent. eosin solution was arranged so as to drip on the centre of a plant laid prostrate on a glass plate, the plant having previously been washed and dried of surplus moisture by means of blotting-paper (Fig. 2).

The rate of spread of the eosin solution to the tip and base respectively, viz. in the succubous and incubous directions, was noted for more than thirty plants with the following typical results:

TABLE III

Plant No.	Succubous portion. Total length in cm.	Succubous portion. Distance travelled by eosin per min. in cm.	Incubous portion. Total length in cm.	Incubous portion. Distance travelled by eosin per min. in cm.
1	3·1	3·1 (tip)	3·1	0·9
2	2·9	2·9 (tip)	2·9	0·55
3	3·7	3·7 (tip)	3·7	0·8
4	3·0	3·0 (tip)	3·0	0·6
5	2·7	2·7 (tip)	2·7	0·7

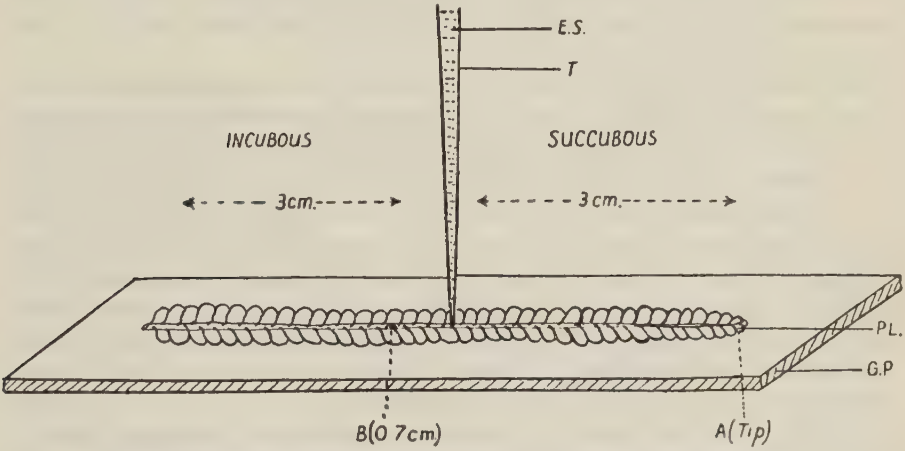

FIG. 2. Diagram of apparatus used in experiment, G.P, glass plate; PL., plant; T., tube; E.S., eosin solution. The figures given are typical results of an experiment, and A. and B. the points reached by the stain in the succubous and incubous directions respectively in one minute.

It is obvious from these figures that the rate of external water conduction is far more rapid in the succubous than in the incubous direction in this plant, and there seems to be no valid objection to the assumption that the same will be true for all other foliose forms, although, on account of the much smaller size and the relative complexity of branching, &c., in most other forms available, no measurements were taken with any but the above species, the larger size and simplicity of which made it a suitable type for experimental purposes.

These observations were now correlated with a study of the natural habitat of a number of succubous and incubous forms. As many species were collected as possible, and from the greatest variety of habitat obtainable locally. As a result it was observed that the succubous types grew in habitats where there was an abundance of ground or surface water, which would be supplied from below and would travel up the stems. For example, *Plagiochila* *asplenioides* var. *major* (Garnant, Glam.), and *P. asplenioides* var. *devexa* (Llangunnar, Carmarthenshire) were found in damp shaded banks, the plants totally surrounded by grass and moss. *Lophocolea heterophylla* (Golden Grove, Carmarthenshire), *L. bidentata* (Swansea, Glam.), and *L. cuspidata* (Alltygrug, Glam.) grew on moist hedge banks, while the last-named species invariably grew in association with a moss. *Alicularia compressa* (Swansea Valley, Glam.) inhabited the wet banks of mountain streams, while *A. scalaris* (Swansea Valley) grew on damp shaded banks. *Saccogyna viticulosa* (Darren Mountain, Swansea Valley), *Gymnocolea inflata* (Swansea Valley, Glam.), *Geocalyx graveolens* (Alltygrug Mountain, Glam.) all grew on shaded hedge banks where there was an abundance of ground water. *Scapania nemorosa* (Cray, Breconshire), *S. undulata* (Golden Grove, Carm.), *Chiloscyphus polyanthus* (Cray, Breconshire) all grew on the sides of streams where again the water was obviously supplied from below.

Incubous forms, on the other hand, grew in habitats where the water supply came from above, so that the water must travel from the tip to the base, i.e., in a direction down the stem which would represent a succubous arrangement; for example, *Frullania dilatata* var. *microphylla* (Golden Grove, Carm.) was found growing vertically on a tree trunk, approximately four feet above the ground, the water supply being clearly derived from rain, &c., trickling down the trunk, *F. Tamarisci* (Rhossilli Downs, Gower.) was found growing in dense patches on large rocks, the water supply being obtained from rain and sea mists, i.e. from above. *Madotheca cordeana* (Castel Coch, Cardiff) and *Catypogeia fissa* (Ty Mawr, Carm.) grew on steep sides of ditches, while *Madotheca platyphylla* (Llarnarthney, Carm.) grew on the sides of walls several feet from the ground. All these incubous types therefore occupy a relatively aerial position, and must obtain their water supply directly from above, so that even in incubous forms external transport of water takes place in a succubous direction.

The conclusion to be drawn from the observations recorded is that the relative aerial habitat of the foliose Hepaticae of the incubous type with a supply of water from above, and the terrestrial habitat of those of the succubous type with an abundant supply of ground water, represents a distribution ensuring the maximum possible water supply to both, since in both types the water passes as external films over the plants in a succubous direction. The transport in this direction has been shown to be much faster than in the incubous direction.

The writer wishes to acknowledge with thanks all help and advice given by Dr. F. A. Mockeridge.

The Effect of Ascorbic Acid and Certain Indole Derivatives on the Regeneration and Germination of Plants

BY

W. DAVIES

G. A. ATKINS

AND

P. C. B. HUDSON

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I. Previous Work

ASCORBIC ACID (vitamin C) has been known for some years as a necessary constituent of animal food. It is the antiscorbutic vitamin present in the fruits and leaves of certain plants, especially the citrus fruits. Its role in the plant is unknown, though recently a few workers have begun investigations into the part it plays in plant processes.

Subsequent to the discovery of a growth hormone in plants by F. W. Went (1928), and its isolation by Kögl (1932) under the two forms auxin α and auxin β, a third substance having similar properties was found in urine and given the name *Heteroauxin*. This is identical with the synthesized compound known as β-indolylacetic acid, and forms, along with certain homologues (β-indolylpropionic acid and ω-skatolylmalonic acid (which yields the former by loss of carbon dioxide), and the kindred β-ethylindole), the subject of the present investigation. In order to estimate the role played by the vitamin, parallel experiments with growth compounds were carried out in all cases, the results being found fully to justify this procedure. Recent work may be summarized as follows:

Laibach and Maschmann (1933) claim that a growth-promoting compound is present in the juice of oranges, lemons, &c. They tested these juices by

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the Went method on Avena and obtained a positive result, though whether it was the ascorbic acid present which gave this response they did not determine.

Carter (1935) tested a low concentration of orange and other fruit juices on the growth of gametophytes of *Laminaria saccharina*. He found that the addition of orange juice shortens the period of germination, and proposes to try pure ascorbic acid. Whereas normally the greatest growth in the sporophytes is found in those on the surface of the solution, when the juice is added it is found in the submerged ones.

Hansen (1935) claims that vitamin C causes an increase of 35–75 per cent. in the dry weight of plants, the greatest difference occurring during the flowering period. The treated plants also showed a much higher content of vitamin C. The increase is specifically due to ascorbic acid, and not merely to an addition of organic material to the inorganic medium, since a similar addition of glucose effected no increase in the growth of the plants.

Havas (1935) experimented with the germination of seeds and seedlings in vitamin C. Using concentrations of 1/10,000 and 5/10,000 he found no stimulation of germination, but an acceleration of growth and an increase of 25–30 per cent. in the length of the shoots. The weight of the shoots increased likewise 25–30 per cent. and the roots 50 per cent. A concentration of 2·5/1,000 exercised a slight inhibitory effect upon germination and a very marked one (24–45 per cent.) upon the growth and weight of the seedlings. The concentration of 5/1,000 proved to be practically lethal.

Oats were much less sensitive both as regards the stimulatory and retarding effects of the ascorbic acid. On the other hand, it was noticed that upon seedlings of plants—such as tomato and paprika—which have naturally a high vitamin C content, a concentration of 5/10,000 was enough to exercise inhibitory effects.

Adult tomato plants treated with a 1/10,000 concentration of ascorbic acid showed no acceleration of growth and flowering, but although the number of fruits produced was less than in the controls, their total weight was 20 per cent. greater. Titration of the vitamin C content of the treated seedlings and tomato plants revealed no increase as compared with the controls.

Laibach (1933) found that the constituents of a water extract of pollen and an ether extract of human urine stimulated the formation of roots on the cut stems of a number of plants.

Thimann and Koepfli (1935) claim that heteroauxin is fully active in promoting root-formation in peas, and suggest the identity of growth-promoting and root-forming substances. Indole and certain homologues of heteroauxin are inactive in root-formation.

Went (1934) suggests a method analogous to the Avena unit for the curvature of coleoptiles, by which the quantity of root-forming compound can be measured.

Laibach (1933 *a*) caused decapitated epicotyls of *Vicia faba* and various leaf stalks to grow in thickness by placing on them the pollinia of orchids, which he shows to exude large quantities of auxin. He did not study the anatomical changes involved.

Snow and Le Fanu (1935) found that extracts of urine caused cambial growth and roots in strips of decapitated sunflower hypocotyls.

Snow later (1935 and 1935*a*) found that pure auxin α and pure synthetic heteroauxin bring about strong cambial growth. Thus the same hormones which elongate stems and coleoptiles and activate root-formation, also cause growth in thickness by division of cambial cells.

Schuck (1935) found that lettuce leaves contain a growth inhibitor which diffuses from them, and prevents germination if in sufficient amount.

Tincker (1935) has recently published some experiments in which samples of oestrin and an extract of yeast containing auxin were added to distilled water in which hyacinth plants were growing. He found no stimulation of growth or flowering, though the auxin caused great bacterial development.

II. SOURCE AND METHOD OF PREPARATION OF CHEMICAL COMPOUNDS USED

The organic chemicals used have been prepared or obtained in as high degree of purity as possible. Our first experiments with vitamin C were carried out with pure vitamin C or laevo ascorbic acid prepared from paprika by British Drug Houses Ltd. In our later experiments we used the synthetic material supplied by the same firm, and comparative experiments carried out by us show that there is no appreciable difference in phytochemical potency between these two forms of vitamin C.

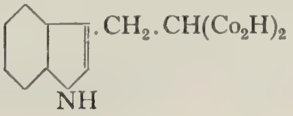
β-Indolylacetic acid (heteroauxin) was prepared in good yield after the method of Majima and Hoshino (1925). The pure colourless crystals from benzene melt at 167·5 to 168·5° C. (Majima and Hoshino give m.p. 164·5–165° C.), and this solid material can be kept unchanged for many months, whereas less pure material or the pure substance in solution decomposes on standing to form skatole.

β-Indolylpropionic acid prepared in the following way is free from contamination with the, in some respects far more physiologically active, β-indolylacetic acid (see Thimann and Koepfli, 1935), and has m.p. 132–3° C.

ωω-Diethoxypropyl diethylmalonate (I), prepared by the interaction of β-chloropropionacetal and the sodium derivative of diethylmalonate (II), is converted into the oily phenylhydrazone (III) of the corresponding aldehyde, and the product of the Fisher indole synthesis isolated as ω-skatolylmalonic acid (IV). This on heating yields β-indolylpropionic acid, which unlike β-indolylacetic acid is relatively stable to heat at 180° C. (Also see Manske and Robinson (1927).)

$$(OEt)_2CH.CH_2.CH_2.CH(Co_2Et)_2 \qquad (I)$$

$$Ph.NH.N = CH.CH_2.CH_2.CH(Co_2Et)_2 \qquad (III)$$



(IV)

Attempts to overcome the drawback of the low activity of the chlorine in β-chloropropionacetal have been made by converting it (with sodium iodide and acetone) into β-iodopropionacetal, which is not obtained in a pure state, distillation yielding considerable quantities of iodoform. It is also found that (II) does not add on to acrolein acetal at 112–145° in a sealed tube to form (I).

The neutral hydrazone (III) separates as a viscous oil when (I) is boiled with phenylhydrazine in dilute acetic acid. The purified and dried hydrazone gave an indole derivative by using as condensing agent hydrogen chloride in alcohol, or hydrogen chloride or zinc chloride in boiling cumene, but in all these cases the yield of β-indolylpropionic acid is small. The carefully controlled use of sulphuric acid in alcohol brings about the conversion of (I) into the ester of (IV) in one operation, and an overall yield of β-indolylpropionic from (I) of 62 per cent. is obtained.

β-Ethylindole (b.p. 149–153°, 17 mm., m.p. 37°) is obtained in almost theoretical yield by refluxing and mechanically stirring for five hours equal weights of η-butylaldehyde phenylhydrazone and powdered zinc chloride in xylene. (Compare D.R.P. 238,138.) Similarly 2-phenylindole has been prepared giving 90 per cent. yield using superheated steam distillation to work up the product.

Method in detail of preparation of β-indolylpropionic acid.

β-Chlorpropionaldehyde acetal is made (see 'Organic Syntheses', vol. xi, p. 29), better mixing being found to occur when the end of the dropping tube for the acrolein was above and not below the surface of the alcohol. 'Molecular' sodium (4·2 gm., 2 atoms) in xylene (150 c.c.) is dissolved by refluxing for 1½ hours with diethylmalonate (29 gm. : 2 mols.), β-chlorpropionaldehyde acetal (15 gm. : 1 mol.) is then added and the mixture refluxed for nine hours. The presence of 100 per cent. excess of (II) expedites the reaction and results in the formation of no appreciable quantity of acrolein acetal. Most of the xylene is distilled off, the residue treated with water, carbon dioxide passed, extracted with ether, and dried over sodium carbonate. The ether acetal (I) boils on refractionation at 152–153 °/0·5 mm. and weighs 8 gm. This quantity is mixed with phenylhydrazine (4 gm.) in absolute alcohol (100 c.c.) and treated with concentrated sulphuric acid (2 c.c.) and boiled for three minutes, when the originally yellow solution becomes green and finally brownish yellow. At this stage the hydrazone (III) is formed, and initial addition of an excess of sulphuric acid is apt to stop hydrazone formation through the separation of phenylhydrazine sulphate. The solution is now refluxed for 2½ hours with a further 8 c.c. of concentrated sulphuric acid, 150 c.c. water added, and the cold liquid treated with concentrated hydroxide solution until the whole is about 2·5 N . NaOH. The indole malonic ester derivative is hydrolysed by refluxing for an hour, the product is poured into excess water, acidified with hydrochloric acid, and repeatedly extracted with ether. Removal of the dried ether left the heat-unstable *ω-skatolyl-*

malonic acid (IV), which may be purified by dissolution (below 100°) in anisole, followed by precipitation with petroleum ether in the cold. The colourless needles are excessively soluble in water and soften at 182° and melt with decomposition at 185° and have equivalent 118·3. $C_{12}H_{11}O_4N$ requires equivalent 116·5.

The crude malonic acid is heated in an oil bath at 170–185° until the evolution of carbon dioxide ceases (about 1¼ hours) when 4 gm. (62 per cent. yield from I) of crude β-indolylpropionic acid are obtained. It separates from petroleum ether (b.p. 80–100°) in stable colourless crystals (m.p. 132–133°). It gives the same colour reactions as β-indolylacetic acid, none of which, however, can be present.

III. EPINASTIC RESPONSE OF TOMATO AND CASTOR-OIL PLANT (RICINUS)

Petioles of tomato and castor-oil curved readily downwards on the application of β-indolylacetic acid and β-indolylpropionic acid to the upper side near the stem, but no effect was obtained from the other indole derivatives.

The results obtained are in course of publication elsewhere. The procedure followed was mainly that given by Zimmerman, Crocker, and Hitchcock (1935), though other methods were also developed. As ascorbic acid had been found to stimulate growth, an attempt was made to induce nastic curvature by its application to the petiole.

Ascorbic acid is soluble in water but not in oil, so that solutions in lanoline (cf. Zimmerman et al (1935)) could not be used. A method of placing drops of water solution (1 part in 1,000) in the axils of tomato branches was tried, but gave no result. Usually it escaped, but otherwise the droplets clung to the hairs and did not touch the branch, or rapidly evaporated. Washing with absolute alcohol was tried and caused wetting of the petioles. Still no response occurred, though it was tried six times and the solution was applied at short intervals for periods of nine hours. Various improvements of method were then introduced.

(*a*) Small wads of cotton-wool were soaked in the ascorbic acid solution (1/1,000) and applied to the upper side of the petioles close to the main stem. The plant was enclosed in a bell jar to prevent evaporation and ensure humidity. Controls with cotton-wool soaked in glass distilled water were used, and a companion experiment with β-indolylacetic acid (heteroauxin) 1/10,000 was run at the same time. No epinastic bending occurred, though the tomato plants treated with heteroauxin bent readily.

(*b*) The experiment was repeated with another series of plants, but this time the wads of cotton-wool were replaced by newly soaked ones every half-hour for a period of six hours. No response occurred, though on further repetition with fresh supplies of vitamin C solution (1/1,000) a slight tendency to epinasty in the young leaves was noticed. This passed off later and seemed to possess no significance, unless it was a toxic effect.

(*c*) As vitamin C rapidly oxidizes attempts were made to prevent this occurring. Equal quantities of water solution (1/1,000) and 3 per cent. agar were mixed, and blocks so formed were placed on the petioles with 12 per cent. gelatine. The two plants treated were kept under humid conditions, but after 24 hours were still the same as the controls.

(*d*) As the diffusion from the agar would be slight, cotton-wool was taken and boiled to exclude air, and then surrounded by cups of paraffin so that only one side was exposed. They were then dipped in 1/1,000 water solution and placed on the petioles. No curvature had occurred after 12 hours. Similar cotton-wool was boiled and soaked in the solution and then fastened on the petioles with an air-tight rubber bandage. Again no curvature resulted.

(*e*) It was decided to use a mixture of vitamin C and adrenalin in water, so that any oxygen present would be taken up by the adrenalin and the vitamin C thus shielded. Ascorbic 1/1,000, and adrenalin 1/1,000 and 1/10,000 were used. The cotton-wool was boiled to exclude air and then dipped in the solution. Two plants for each strength of adrenalin were used, and also controls with water and adrenalin respectively.

No epinastic response occurred.

(*f*) Castor-oil plants gave good epinastic responses to heteroauxin, and as it was thought that the tomato might have powers of rapid translocation of vitamin C, a kindred series of experiments was run with Ricinus. No curvature resulted. Cotton-wool, soaked in 1/1,000 vitamin C, was placed along one side of the castor-oil stem, and a control done with water; but no sign of bending was noticed.

It was decided that ascorbic acid had no power of producing nastic response by any method of asymmetrical application to the petiole. Possibly the plants possessed rapid powers of translocation, for general growth effects showed that penetration of the epidermis by the vitamin was occurring. No attempt was made to estimate the quantity of ascorbic acid in the plants, and beyond noting a slight increase in size no measurement of dry weight was made. Evidently the curvature of the Avena coleoptile which Laibach (1933 *a*) obtained with lemon juice was not due to the vitamin C contained. Experiments carried out on oats using the Went technique also gave negative results for ascorbic acid, though the normal response to heteroauxin was obtained.

IV. Regeneration of Buds and Roots in Willow Branches

A representative range of willow branches cut to 12-inch lengths was taken, and eight of corresponding thicknesses and from similar regions of the tree were used with each of the types of solutions specified. The eight branches were placed inside glass containers so that they stood vertically with their morphologically lower ends downwards, and the jars were sealed with glass and vaseline. Another series of four branches were totally immersed in the respective solutions for 48 hours in a horizontal position, and were then marked with a cotton thread and enclosed in the glass jars with the others.

A quantity of inorganic nutrient solution was made up in glass distilled water according to Pfeffer's (1906) formula (see Knudson, 1922):

$Ca(NO_3)_2$	0·8 gm.
K_2HPO_4	0·2 gm.
$MgSO_47H_2O$	0·2 gm.
KNO_3	0·2 gm.
KCl	0·1 gm.
$FeCl_3$	8·0 gm.

Glass-distilled water, 1 litre.

The various organic compounds tested were dissolved in 200 c.c. of this stock solution, and a control of the same was used.

(*a*) In the first experiment the following solutions and concentrations were used:

Ascorbic acid	1/1,000
Ascorbic acid	1/10,000
β-Indolylacetic acid	1/10,000
β-Indolylacetic acid	1/100,000
β-Indolylpropionic acid	1/1,000
β-Indolylpropionic acid	1/10,000
ω-Skatolylmalonic acid	1/1,000
ω-Skatolylmalonic acid	1/10,000
β-Ethylindole	1/10,000
Control.	

The first appearance of roots and shoots was given by ascorbic acid 1/10,000 on the sixth day, but as the experiment continued the initial difference to some extent disappeared. No advantage was shown by the branches which had been completely soaked for 48 hours, and this was not repeated in subsequent experiments. Striking polarity of the willow branches occurred, in that the thicker branches produced evenly distributed roots, and the thinner branches shoots. Occasionally a thick branch produced a shoot also at the upper end, and roots at the base of the thin branches were common, but these occurred at a later stage. Basal roots which grew within the solution were longer than those in air in the case of the ascorbic solutions and the control, but very short and rare in the indole compounds.

The result after fifteen days was tabulated in order of size as follows:

(i) β-Indolylacetic acid	1/100,000
(ii) Ascorbic acid	1/10,000
(iii) Ascorbic acid	1/1,000
(iv) β-Ethylindole	1/10,000
(v) β-Indolylpropionic acid	1/10,000
(vi) ω-Skatolylmalonic acid	1/10,000
(vii) Control.	
(viii) ω-Skatolylmalonic acid	1/1,000
(ix) β-Indolylacetic acid	1/10,000
(x) β-Indolylpropionic acid	1/1,000

Three definite groupings of size have been suggested by the brackets, though in the case of numbers (ix) and (x) no roots or shoots at all were produced.

The β-indolylacetic 1/100,000 and ascorbic 1/10,000 gave much the same extent of development. Whereas roots and shoots had appeared first on the ascorbic, the roots were now longer in the β-indolylacetic and the shoots longer in the ascorbic.

The result appeared paradoxical, in that while the compounds used were clearly stimulating to growth as compared with the control, the lower concentration of each substance was more effective than the higher. Especially was this the case with heteroauxin (β-indolylacetic) itself, since this at the one time was top and bottom of the list.

Although the original nutrient solution had been sterilized by autoclave, the various containers became infected during the arrangement of the willow branches, and fungi grew copiously in the ascorbic 1/1,000, and slightly in the ascorbic 1/10,000 and β-indolylacetic 1/10,000. The falling-off in growth in these solutions might be due to toxic products of the fungi, and the lag which had occurred in the ascorbic after the first appearance of growth might be due to oxidation of the compound. It was decided to confine subsequent experiments to a few of the principal compounds, and to test these in series of equal concentrations, ending the experiments within eight days in all cases. Precautions against infection were also taken.

(*b*) Concentrations of 1 in 100,000 were first used, and the compounds ascorbic, β-indolylacetic, and oestrin investigated. The last was incorporated because Hoffmann (1934) reported an acceleration of the growth of cereals through its use. It was obtained in sealed tubes from British Drug Houses Ltd., and was considered pure. When tested on the petioles of tomato plants or on oats by the Went (1928) method it gave no curvature, and so probably contained little auxin impurity.

Fig. 1 shows the experiment eight days later. Four typical branches have been selected from each container out of the twelve tested, and these have been arranged in four groups. The extent of penetration of the solution by the branches is shown by a slight discoloration at the lower end. The small white areas are lenticels, and appeared about 72 hours after the commencement of the experiment. From these develop the roots, but the shoots appear in the axils of the original leaves.

From the figure can be seen the copious development of roots and shoots in the ascorbic acid. Some roots are actually growing within the solution. The β-indolylacetic is next, and the control and oestrin about the same. Lenticels were numerous on the branches in the β-indolylacetic, even on the thinner ones which normally give shoots. It seems especially effective in the stimulation of roots. No special effect of the oestrin was apparent at this stage, though in subsequent stages it showed a slight precedence over the control. Any claims it may have as a growth promoter of plants can obtain no support from this series of experiments.

It was usual, when the experiments were continued longer than eight days, for the β-indolylacetic to overtake and surpass the effect of the ascorbic. Fig. 2 shows one of the many repetitions of this experiment on the 10th day. The ascorbic still has longer roots than the β-indolylacetic, and has a much greater development of shoots, but the roots from the branches in heteroauxin grow out two or three from the same point. By the 15th day the roots in ascorbic had been surpassed by the other. This figure shows the customary exaggerated root growth within the solution in the case of ascorbic acid, and the absence of roots beneath the level of the liquid in heteroauxin.

When the relative efficiency of ascorbic and β-indolylacetic as growth promoters had been established by numerous experiments, the other compounds β-ethylindole and ω-skatolylmalonic were tested. These, as previously stated, gave no epinastic response with the tomato or curvature of oats by the Went (1928) test. The higher homologue of heteroauxin, β-indolylpropionic acid, which yielded a good epinastic response but no curvature on oats, was also used.

They were found to be slightly effective in growth promotion, as the willow branches tested surpassed the controls in root formation, but could not be compared with β-indolylacetic or ascorbic acid. On their efficiency they could be placed in the following order:

1. β-Ethylindole.
2. β-Indolylpropionic acid.
3. ω-Skatolylmalonic acid.

That some of them should fail to induce curvature or epinasty and yet show slight effect in growth-promotion was interesting, in consideration of the fact that slight differences in chemical constitution are usually associated with total difference in physiological effect.

(*c*) The effect of lower concentration of the growth compounds was now tried, and solutions of a strength of 1 in 500,000 used. Whereas with the former concentration roots had appeared on the branches in ascorbic in six or seven days, they now appeared on the 9th day, while the other compounds produced them on the 10th day. The result at this very low concentration was as follows:

1. Ascorbic acid.
2. β-Indolylacetic acid.
3. Control.
4. β-Indolylpropionic acid.

Evidently the stimulatory action of the ascorbic acid persisted, but it was difficult to discriminate between the control and the indole derivatives. If anything, heteroauxin had a slight positive effect and β-indolylpropionic a slight retarding effect, but the difference was so slight that it could easily be overlooked. The other compounds seemed to have lost any power of stimulation at this concentration.

Considering that heteroauxin still showed considerable physiological activity in inducing curvature in oats at a concentration of 25×10^{-6} (Kögl and Kostermans, loc. cit.; Thimann and Koepfli, loc. cit.), its loss of efficiency as a growth promoter in these experiments was surprising.

(*d*) The effect of high concentration of the compounds was now tried with a strength of 1 in 2,500. While roots appeared on the control branches in 10 days, those in the heteroauxin were delayed until the 14th day and those in ascorbic until the 16th day. The result was as follows:

1. Control.
2. β-Indolylacetic acid.
3. Ascorbic acid.
4. β-Indolylpropionic acid.

The growth of roots on the control branches was far more than on the others though the ascorbic was definitely inferior to the heteroauxin, and the β-indolylpropionic showed no growth at all. No lenticels even appeared on the last-named branches.

These experiments fully support the paradoxical result of experiment (*a*) in which the lower concentrations of the various compounds were more efficient as growth promoters than the higher. High concentration evidently retards growth. It appeared that ascorbic acid was more stimulating than heteroauxin in low concentration, and more retarding in high. The result for ascorbic acid could be compared with the familiar hyper effect of vitamins on animals. Whether the indole derivatives act in similar fashion as the animal vitamins, so that retardation of growth follows from excess stimulation, or act by affecting in some way the translocatory system, is uncertain.

V. Germination of Seeds

It was decided to germinate seeds and grow seedlings in solutions to which the various compounds had been added. Pfeffer's (1906) inorganic nutrient solution was used, and the compounds were made up in varying concentrations for a preliminary experiment. Oats (*Avena sativa*), cress (*Lepidium sativum*), and mustard (*Brassica alba*) were found to germinate and grow at much the same rate and were used in the series of experiments. Sterile conditions were maintained throughout, Petri dishes being used containing three filter-papers and 10 c.c. of solution, while ten of each type were distributed evenly in the interior. The glumes were removed from the oat seeds. The Petri dishes were of sufficient height to allow the seedlings to grow during the course of the tests.

(*a*) In the first experiment the following compounds and concentrations were used:

Ascorbic acid	1/10,000
Ascorbic acid	1/100,000
β-Indolylacetic acid	1/10,000
β-Indolylacetic acid	1/100,000
β-Indolylpropionic acid	1/1,000
β-Indolylpropionic acid	1/10,000
ω-Skatolylmalonic acid	1/1,000
ω-Skatolylmalonic acid	1/10,000
β-Ethylindole	1/10,000
Control.	

At the end of 36 hours germination had progressed sufficiently to show the size of the developing embryo, and this was found to be proportionately the same as the seedlings after 60 hours, when the experiment was concluded. Havas (1935) found that with vitamin C and his controls, the rate of germination was the same and differences occurred during later growth. This was not the case in these experiments, and to counteract the initial advantage gained in germination by certain of the cultures, later tests were devised in which the growth compounds were added after the 36-hour period of germination had elapsed.

The rest of the preliminary test with the above compounds was as follows.

(1) Ascorbic acid	1/100,000
(2) Ascorbic acid	1/10,000
(3) Control.	
(4) β-Indolylacetic acid	1/100,000
(5) β-Ethylindole	1/10,000
(6) ω-Skatolylmalonic acid	1/10,000
(7) β-Indolylacetic acid	1/10,000
(8) β-Indolylpropionic acid	1/10,000
(9) ω-Skatolylmalonic acid	1/1,000
(10) β-Indolylpropionic acid	1/1,000

It was noteworthy that in all cases the weaker concentration was more efficient or less retarding as the case may be. Only the ascorbic acid proved stimulating to growth, all the indole derivatives retarding it, but between the two concentrations of ascorbic was a marked difference. Brackets have been inserted to suggest groupings as to size of seedlings. In the case of the last compound, β-indolylpropionic 1/1,000, no germination or growth at all occurred, the concentration evidently being lethal. A marked pigment effect was noticed in the mustard, the anthocyanin red being very prominent in ascorbic and heteroauxin, and entirely absent in the β-ethylindole.

(*b*) It was decided to run a restricted series of experiments, using a concentration of 1 in 10,000 of the compounds. Four compounds were chosen as follows, the others being tested later:

Ascorbic acid.
β-Indolylacetic acid.
β-Indolylpropionic acid.
β-Ethylindole.

In Fig. 3 is shown the result of the first of these experiments after 60 hours, the seedlings being arranged in separate transverse rows of oat, mustard, and cress. Pairs of seedlings have been taken for purposes of photographic record from the various dishes, and those grown in the four compounds and the control are in vertical rows. From left to right they represent:

(1) Ascorbic acid.
(2) β-Ethylindole.
(3) β-Indolylpropionic acid.
(4) β-Indolylacetic acid.
(5) Control.

It will be seen that those grown in ascorbic are much larger than the controls, and that those in heteroauxin are the smallest. Notice the small roots of the seedlings grown in β-indolylacetic and β-indolylpropionic.

Other indole compounds, β-methylindole and ω-skatolylmalonic, were introduced in subsequent experiments. The first was found to retard growth slightly less than β-ethylindole, so that it ranked next below the control. It likewise prevented anthocyanin pigment formation. ω-Skatolylmalonic came between β-ethylindole and β-indolylpropionic. In order of growth-inducing and germinating properties the compounds can then be ranked as follows:

(1) Ascorbic acid.
(2) Control.
(3) β-Methylindole.
(4) β-Ethylindole.
(5) ω-Skatolylmalonic acid.
(6) β-Indolylpropionic acid.
(7) β-Indolylacetic acid.

The roots of the seedlings grown in heteroauxin and β-indolylpropionic were very short and thick at the base and cone shaped. (See Fig. 6.) They were quite unable to support the cress and mustard plants, though the large seed of the oats gave stability so that the coleoptile could become vertical.

The cress and mustard seedlings were usually prostrate in these solutions, and the hypocotyls lengthened abnormally. Fig. 6 shows mustard plants grown in β-indolylpropionic acid (1/10,000). The cone-shaped roots and thick hypocotyls can be seen. The lengthening of the hypocotyls was evidently the result of direct contact with the solutions, since oat coleoptiles which fell prostrate also elongated considerably in comparison with those that remained vertical. It was remarkable that prostrate seedlings lost all powers of orientation, and could no longer exhibit normal geotropism.

The occurrence of these thick and stunted roots in heteroauxin is interesting in view of the recent address of F. A. F. C. Went (1935) in which he states that 'Auxin retards the growth of the roots, probably by extending the transverse cell walls'. The appearance of these roots certainly suggests growth in width rather than length.

(*c*) To separate the effect of the compounds on germination and subsequent growth, a series of experiments was now run in which the seeds were germinated in the culture solution and the compounds added after a 36-hour period. Ten cubic centimentres of Pfeffer's inorganic culture solution was placed in each of six Petri dishes along with the usual number of filter-papers, and ten seeds of oats, cress, and mustard were evenly spaced in the dishes in the usual manner. After 36 hours all the seeds had germinated and were all at precisely the same stage of development and the same size.

The five compounds used in this experiment were then added at a concentration of 1 in 10,000 in glass-distilled water. Ten cubic centimetres of each solution were poured evenly over the surface of the filter-papers without disturbing the seedlings. This meant a final concentration of 1 in 20,000, half the concentration of the nutrient solution, and double the quantity of liquid. To ensure that the result could be compared safely with the previous experiments, these were repeated with altered concentrations and amount of solution and no appreciable difference detected. Within certain limits the quantity of solution did not affect the growth of the seedlings, and the lower concentration still preserved the same ratio of development in the various compounds. The retarding effect of the indole derivatives was not quite so exaggerated with half the concentration, but the stimulating effect of ascorbic acid remained undiminished.

The result at the conclusion of sixty hours was as follows:

(1) Ascorbic acid	1/20,000
(2) Control	„
(3) β-Methylindole	„
(4) β-Indolylacetic acid	„
(5) β-Ethylindole	„
(6) β-Indolylpropionic acid	„

In Fig. 5 a pair of each kind of seedlings in the dishes has been photographed for purposes of comparison. Again the roots in β-indolylacetic and β-indolylpropionic are shorter and thicker than the others. The plants in ascorbic are much larger than the control, whereas those in β-methylindole and β-indolylacetic are of much the same size. This result was maintained in all repetitions of this experiment.

Apparently even when the compounds are applied subsequent to germination considerable differences in growth ensue. It is not merely the initial differences in rate of germination which determine the end result, but the influence of the compounds persists throughout. The only divergence in the ratio of efficiency between the various compounds is shown by heteroauxin itself, which, instead of showing least growth, is now practically equal in size to the control. Thus when seeds are germinated and grown in β-indolylacetic they are retarded more than is the case with any of the other indole compounds; when it is applied after 36 hours the growth is little retarded. To throw more light on this anomaly other experiments were necessary.

(*d*) Instead of adding the compounds 36 hours after the commencement of the experiment, two series of experiments were run with the compounds added at 24 and 48 hours respectively. No clear sign that germination had begun was apparent at the 24-hour stage, while after 48 hours the seedlings had grown slightly in size. The method, apart from the time of application of the compounds, was exactly the same as had been previously described. Apart from the effect of the plants in heteroauxin no changes in ratio of growth occurred.

When β-indolylacetic acid was added at the end of 24 hours the resulting growth at the end of 60 hours was intermediate between that where it was present during the whole period and that where it was applied at the end of 36 hours.

It was comparable with the result for β-ethylindole in Fig. 5.

When applied at the end of 48 hours growth was slightly more than in the case of the control. (See Fig. 4.) In both cases the small size of the roots, however, was noticeable, the amount of general growth being roughly proportional to this. The apparent anomaly in the case of heteroauxin, which at first glance might make it appear to be a retarding factor specific to germination, could clearly be explained in terms of root development. The above experiments showed its retarding influence most potent when applied during germination, less so when applied after 24 or 36 hours, while it was slightly stimulating after 48 hours. Taking for granted that there is a rough correlation between size of root and shoot, since nutrient materials absorbed by the former determine to some extent the possibility of growth in the latter, even in the seedling, it can be seen that when heteroauxin prevented root growth from the beginning the shoots would be correspondingly short, whereas when it was applied after the development of roots of a certain length as at the 24-, 36-, or 48-hour periods, the growth of the coleoptile and hypocotyl would vary proportionately. Seedlings which fell prostrate, so that the shoot made direct contact with the solution, were always abnormally long. Possibly the extent of translocation of heteroauxin from root to shoot determined its size. The Went–Cholodny hypothesis that the auxins increase the longitudinal growth of shoots, but decrease that of roots is, of course, assumed in this explanation.

The other indole derivatives did not show such marked effects as did β-indolylacetic, since their efficiency, if any, in inducing longitudinal growth of shoots is only slight in comparison with heteroauxin. β-indolylpropionic acid, which caused only moderate epinasty of tomato petioles in our experiments, showed marked retardation of root development, and can be compared throughout in limited degree with the much more efficient heteroauxin.

(*e*) Experiments were now run in much lower concentration of the compounds, that of 1 in 100,000 being used. The same method was followed as in the (*b*) series of experiments, the seeds being both germinated and grown to the 60-hour period in the compounds.

The resulting growth was in the following order.

(1) Ascorbic acid.
{(2) Control.
{(3) β-Indolylacetic acid.
(4) β-Indolylpropionic acid.

Even at such a low concentration ascorbic showed great powers of stimulating growth, the original experiment (*a*) being fully confirmed. Heteroauxin was still retarding in effect, though not to such an extent as β-indolylpropionic. As has been already suggested, this does not necessarily mean that the latter is more toxic than heteroauxin, but that the balance of growth, occurring after positive effect on shoot and negative on root, is in favour of β-indolylacetic.

At a concentration of 1 in 500,000 ascorbic was still slightly stimulating, and β-indolyl acetic and β-indolylpropionic were still slightly retarding. Evidently there is no limiting concentration at which the balance of positive and negative effects is in favour of stimulation. The roots still showed considerable retarded development.

(*f*) Seeds were germinated in a high concentration of the compounds, viz. 1 in 1,000.

The resulting growth was as follows:

(1) Control.
(2) Ascorbic acid.
(3) β-Indolylacetic acid.
(4) β-Indolylpropionic acid.

The ascorbic had now definitely a retarding effect, though it was slight. Growth in the indole derivatives had almost ceased, the solutions evidently being almost toxic. There was still a slight growth in the oat coleoptiles, though the roots could hardly be discerned. Some of the oats in heteroauxin fell prostrate in the solution, and these developed coleoptiles four times as long as those on the erect oats.

Evidently high concentration of all the compounds retards growth, though with the indole compounds it is the roots which are especially affected.

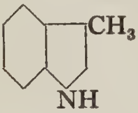
(*g*) An experiment was arranged to test the efficiency of vitamin C as a growth stimulator over a comprehensive series of concentrations, so that the region of maximum effect might be determined more closely. Concentrations of 1 in 500, 1 in 1,000, 1 in 5,000, 1 in 10,000, 1 in 50,000, 1 in 100,000, 1 in 500,000, were used and compared with the control. The result is shown in Fig. 7, where pairs of oat seedlings from the various dishes are arranged in eight groups. Those grown in a concentration of 1 in 100,000 show maximum growth, and there is a steady decrease through increasing concentrations, though even those in 1 in 10,000 are larger in size than the controls. The oats grown in 1 in 500,000 are smaller than the maximum. It is probable that the maximum lies at a 1 in 100,000 concentration, since in all concentrations less than this the growth was less. The greatest care was taken to reduce oxidation of the ascorbic in these solutions, the facility with which this might possibly occur, however, rendering any closer determination of the maximum unwarrantable.

The result was considered sufficient to show that vitamin C exercises stimulating effects on the growth of seedlings in extremely low concentrations, and that in higher concentrations its effect is retarding. The effect is evidently a general one upon the whole plant, since no disturbance of the normal correlation between shoot and root was noticed. It also appeared to maintain a steady effect throughout the period of germination and subsequent growth, at any rate for oats, cress, and mustard. Experiments in progress with different varieties of tomato seedlings are not uniform in result, some of the varieties appearing to germinate without respect to the concentration of ascorbic employed, while others exhibit differences in rate of germination in harmony with subsequent growth effects. Since in these experiments effect of concentration on rate of germination was most strikingly displayed by cress and mustard (those in high concentration not germinating at all), and by oats to a lesser extent, the difference between endospermic and non-endospermic types of seeds might be responsible.

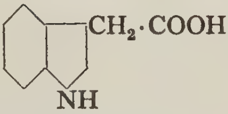
The result with tomato would, however, remove or modify the possibility of such an explanation.

Discussion from the chemical standpoint.

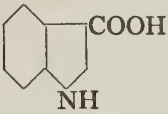
The following compounds closely related to β-indolylacetic acid (II), i.e. heteroauxin, have been prepared and tested.



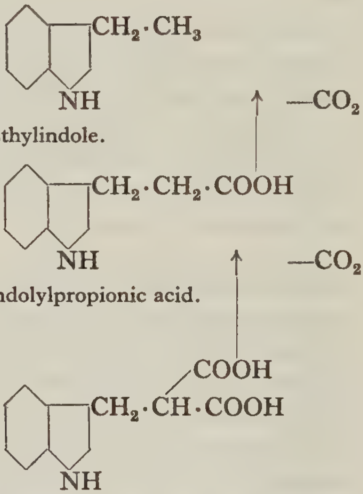
(I) Skatole.



(II) β-Indolylacetic acid (heteroauxin).



(III) β-Indolecarboxylic acid.



(IV) β-Ethylindole.

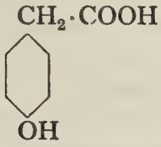
(V). β-Indolylpropionic acid.

(VI)½-Skatolylmalonic acid.

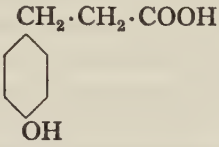
β-Indolylpropionic acid (V) is potent in producing epinastic bending (Hitchcock, 1935), but there is a doubt as to whether (V) gives the oat test (Thimann and Koepfli, 1935), the ambiguity being due to the possibility that the commercial material tested may have contained (II). Our work shows that perfectly pure (V) has no action on decapitated oats in less concentration than 1 mg. in 20 c.c., which is the limit adopted by Kögl and Kostermans (1935). Our further experiments show that in the series of compounds (VI) to (V) to (IV), formed by the successive loss of carbon dioxide, neither (VI) nor (IV) gives the Went test or causes epinastic bending. This indication of the specific nature, as far as the Went test is concerned, of the β-acetic acid group in indole, is correlated with the previously known complete lack of activity of (III) and (I). Kögl and Kostermans also show that the only definitely active derivatives of indole are substitution derivatives of heteroauxin.

It is evident that the normal plant, as opposed to the decapitated oat, does not require the presence of such a restricted or specific type of molecule to bring about a response. For example, Hitchcock (loc. cit.) has shown that not only (II) and (V) but also phenylacetic, and β-phenylpropionic acids cause epinastic bending.

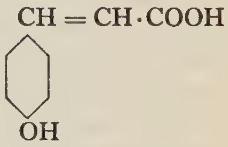
When the formulae of auxin α and auxin β and heteroauxin are considered, it is seen that all three compounds have a carboxyl group, the auxins in addition have hydroxyl groups, whereas heteroauxin has the, in some respects similar, —NH group. It is possible that plants have adapted themselves to the presence of other components of urine than the auxins. The compounds *p*-hydroxyphenylacetic (VII), *p*-hydroxyphenylpropionic (VIII), and *p*-hydroxyphenylcinnamic (IX) have been synthesized. The first two occur in normal urine, they all have some chemical analogies with the three auxins and the above mentioned compounds which cause epinasty, but all three hydroxyphenyl derivatives fail to give the Went test and epinastic bending. It is evident that both these biological actions require a fairly specific type of molecule, and work with other natural products is being continued.



(VII)



(VIII)



(IX)

Another rough chemical analogy has provided interesting results. Vitamin C (*l*-ascorbic acid) and auxins α and β are hydroxy unsaturated aliphatic acids, and a consideration of their formulae shows other structural resemblances indicating that vitamin C may also have phytochemical action. An exhaustive examination, in which all precautions have been taken to eliminate the danger of the destruction of vitamin C by atmospheric oxidation during the experiments, clearly shows that vitamin C causes no bending in the decapitated oat or the normal plant; on the other hand, very striking evidence of the promotion by vitamin C of root-formation and germination has been obtained. The optimum concentration is about 1 in 100,000, much stronger solutions actually hinder germination, whereas 1 in 500,000 gives a positive effect. Heteroauxin and β-indolylpropionic acid retard germination and root-formation. It is well known that vitamin C occurs in seeds and is apparently produced in the process of germination, and it is accordingly understandable that the addition of slight amounts of vitamin C should improve the initial rate of germination, and that the presence of large amounts should depress this rate.

A list of further chemicals tested for epinastic bending is appended. Almost all the substances are biological products or of biological importance. The substances have not been chosen at random, but after considering the formulae of phytochemically active compounds. As epinastic bending is ascribed to cell elongation and so perhaps to synthesis of cellulose in the cell wall, enzymes which affect carbohydrate formation such as diastase and insulin have been tried, and the work is being continued.

VI. DISCUSSION

The results obtained from the foregoing experiments may for convenience be tabulated as follows before their implications are discussed:

(*a*) Heteroauxin and its homologue β-indolylpropionic acid caused epinastic curvatures of tomato, but this was not obtained with the other indole derivatives or with ascorbic acid.

(*b*) All the compounds were found effective in stimulating root development in willow branches, ascorbic being also effective for shoots. Vitamin C produced the maximum effect, closely followed by heteroauxin and more remotely by the other indole derivatives. Only low concentrations, however, were stimulating, high concentrations being lethal or at least retarding.

(*c*) The effect of ascorbic acid on the germination and subsequent growth of oats, mustard, and cress was stimulating in low concentration, but was retarding in high. The other compounds were retarding throughout, though lethal in high concentration. This was traced to a very evident retardation of root development, direct contact of the medium with the shoot yielding greatly increased growth.

It is clear that the effects shown by ascorbic acid in these experiments differentiate it most definitely from the indole derivatives and presumably from the auxins generally. Its stimulation of growth is evidently not an immediate local effect, since local application does not cause epinasty of tomato petioles or give the Went test on oats. Whatever its role in plant metabolism may be, it can evidently be rapidly translocated, as its effect in all cases is quite general. When applied to tomato petioles the effect is a general growth stimulation with considerable increase in dry weight (cf. Hansen 1935 and Havas 1935), when used with willow cuttings the development of both roots and shoots appears to be hastened, and when used in the germination and growth of seedlings equally beneficial general response occurs. It seems most like in effect to an animal vitamin, resembling it also in the effect of high concentration. Possibly the same role is played in both plant and animal cell, and further investigation of its effects as a phytohormone may throw light on its function as a vitamin.

Of the indole derivatives heteroauxin (β-indolylacetic acid) is found to be the most potent phytohormone of all those synthesized. It caused epinasty of the tomato petiole and gave the Went test of curvature of the oat coleoptile. It stimulated root development in the willow, and was retarding to the germination and growth of seedlings. β-indolylpropionic acid, its homologue, gave a ready epinastic on the tomato, but failed entirely to effect curvature on oats. It was in other respects less efficient than heteroauxin, though clearly of the same type. The other indoles, ω-skatolylmalonic, β-ethylindole, and β-methylindole, gave no epinastic or oat coleoptile response at a concentration of 1 in 1,000, but were slightly stimulating to growth of willow branches and moderately effective in retarding germination.

Bearing in mind that it is usual to find considerable divergence of physiological function and efficiency with closely related chemical structure, the specificity of the auxins for curvature of the oat coleoptile claimed by Kögl (1934) might be accepted. The anomaly of an indole derivative, heteroauxin, apparently possessing precisely the same functions though with an entirely different structure, is not without its parallels in other directions in organic chemistry. But in these other indoles we have compounds which resemble heteroauxin in inducing certain responses, while they fail entirely to elicit others. Its own homologue, β-indolylpropionic, fails only in not yielding the oat test. The others are apparently quite ineffective in both oat and petiole response, but are moderately efficient in the others.

On consideration it appears that at least one of two probable alternatives is possible. Either (i) the various types of response given by the auxins are in no way related or are fundamentally not the same. The response of the oat coleoptile, the epinastic response of the petiole, the stimulation of rooting in willows, and the retardation of germination are not mere modifications of one fundamental growth effect. Or (ii) we are dealing with responses of decreasing specificity. In this sense the oat coleoptile reaction which responds to only one of the indoles, heteroauxin, may be regarded as the most specific, even as it is the most sensitive of all. The epinastic response of the tomato petiole which is given also to the homologue of heteroauxin, β-indolylpropionic, but to none of the other indoles, comes next. The other indoles seem to provoke the quite general response of growth in the willow and prevention of growth in seedlings, and these are evidently the least specific of all. Before weighing the relative merits of these alternatives, further evidence from the experiments must be considered.

The stimulation of root production on willow branches seems closely related to the effect of all the auxins in root production. This is a well-known effect of their application, and Went (1934) has devised a method of quantitatively estimating the efficiency of hormone in a given solution by the growth of roots on pea seedlings. A peculiarity of the willow experiments, and one which raises the major problem of the auxins generally, was that while the development of roots above the solution was stimulated, the growth of roots within the solution was prevented. In the germination experiments the retarding of root development was found to be the main cause of lessened growth. Why should the auxins stimulate the production of roots indirectly, but retard them when in direct contact?

In the germination experiments the roots were definitely retarded in development in the indole solutions. Endospermic seeds, such as oats, do not depend during germination upon the size of the radicle to any great extent, and it was noticed that in high concentration the oat coleoptiles would grow slightly while the non-endospermic mustard and cress hypocotyls showed little growth at all. Apart from the direct curtailment of water and nutrient materials caused in the latter by the prevention of root development, there is also in seedlings generally a process of self-regulation which correlates the size of shoot and root. Hering (1906) and Goebel (1900) showed that a retardation of growth in the root involves a slower growth of the shoot, and this would likewise apply in the case of the oats.

Thus the germination experiments are considered of value only in the retardation of root development which they exhibited. Estimation of the efficiency of the solutions in shoot development would best be investigated without the interference of root correlations by Went's method of cutting off coleoptiles and hypocotyls above the seed, and placing them horizontally in the solutions. Increase in length would then give a measure of the efficiency of the concentration used. From his results it would appear that the relationship between increase in length and concentration is direct for low concentrations only, but does not hold as the concentration increases. Whether there is a maximum above which length again decreases Went (1928) does not state. The coleoptiles and hypocotyls, which in these experiments fell prostrate, were at a maximum length in heteroauxin solutions of 1/10,000, and were considerably shorter in the higher concentrations, but their correlation with the roots might have been the cause.

That the roots were retarded in development because of transverse growth, as F. A. F. C. Went (1935) suggests, seemed to be borne out in these experiments, as Fig. 6 illustrates. We have thus two major problems in the physiological effect of the auxins, one being the fundamental difference in response between shoot and root, and the other the anomaly between direct root retardation and root stimulation at a distance. Perhaps a third problem equally acute could be added, namely, the primacy of the apical shoot through auxin production, and the inhibition of lateral bud and branch development through its diffusion downwards (cf. Thimann and Skoog, 1934), but this falls outside the scope of this paper. The auxins are both phytohormones and organizers, and little is known as yet of the physiology of the latter.

Considering the alternatives previously advanced, it is evident that specificity does exist in the oat coleoptile response, and to a lesser degree in the epinastic response. The germination results can be seen, however, to be modifications of the normal root response. It is possible that the auxins cause one fundamental growth response, a rapid elongation of the cell-wall, and that this has been elaborated and made specific in the tropic and nastic curvatures. In the root the cell-wall elongates in a lateral direction, as do also the meristematic regions of the stem. Root production may be due to stimulation of growing points, or to some blockage in the translocatory system through growth of the meristematic zones. Further research will perhaps throw more light on these major problems.

VII. SUMMARY

New methods of preparation of the indole derivatives ensuring that there is no contamination with heteroauxin are described.

Epinastic response on tomato was obtained with heteroauxin and β-indolylpropionic acid, but not with the other indole derivatives or with ascorbic acid.

The compounds were found to promote the growth of willow branches, ascorbic acid being the most efficient, and heteroauxin next. The other indoles showed decreasing efficiency.

The effect of low and high concentrations was decrease in growth-promoting efficiency, a concentration of 1 in 100,000 being most suitable.

Ascorbic acid also stimulated the germination and growth of seedlings, but in high concentration retarded their development.

All the indole derivatives retarded germination and growth of the seedlings, but this was traced to their retardation of root development.

Abnormal length of coleoptiles and hypocotyls occurred in the case of prostrate seedlings.

At higher and lower concentrations the indole derivatives still retard germination and growth.

APPENDIX A

Preparation of Solutions for Testing

Solutions in water. Glass-distilled water is used throughout. If the finely powdered compound does not dissolve readily in cold water, the thin-walled stoppered bottle and its contents are carefully heated and shaken on the water-bath for a short time care being taken that no crystallization at that concentration takes place on cooling and standing. Very strong solutions of heteroauxin (e.g. 1/500) in water are best attained by the partial neutralization of the acid with sodium hydroxide solution, the final solution not affecting litmus. Dilute solutions of heteroauxin are almost exactly neutral and are best kept in dark bottles.

Solutions of vitamin C are made with special precautions owing to the easily oxidised nature of the compound. The glass-distilled water or Pfeffer's solution is boiled shortly before use, cooled in the absence of air, and a definite quantity used to dissolve the weighed vitamin C (solution A). Solution A is then poured into a bottle with well-fitting glass stopper until the bottle overflows. The stopper is then firmly inserted, leaving no air in the bottle. Solutions of less concentration than (A) can be rapidly obtained by accurate dilution of solution A. When a definite number of experiments have to be made with one concentration of vitamin C it is found to be safer to fill a number of small bottles with this solution and to take one quantity only from each bottle. This eliminates the danger of oxidation which may take place during the process of measuring out from time to time several quantities of solution from a large bottle.

Solutions in oil. The weighed finely powdered compound is shaken with the oil (e.g. olive oil) if necessary up to 115°. Care must be taken that no decomposition of the compound occurs, and the temperature should not approach that of its melting-point if this takes place with decomposition. To dissolve a compound in semi-solid fats such as lanoline, melt the lanoline completely on the water-bath, and stir in the weighed powdered solid. If solution does not readily take place at 100° the lanoline is carefully heated in an oil-bath until spurting takes place (about 115°). The lanoline solution is then poured into a bottle, where it becomes once more semi-solid.

APPENDIX B

Additional Compounds tested on Tomato and Ricinus without inducing Curvature

The test is carried out by placing cotton-wool soaked in the solution (which is aqueous unless otherwise specified), on the petioles.

Compound.	Concentration.
Acetylcholine chloride	1/1,000
Adrenaline	—
Allyl alcohol	—
Benzoylcarbinol	—
Citric acid	—
Dehydracetic acid	—
Diastase	—
Diethanolamine	1/2,000
Ephedrine	1/1,000
Ethanolamine	1/2,000
β-Ethylindole	1/10,000
β-Ethylindole	1/1,000 in olive oil
Glucosaminehydrochloride	1/1,000
Hippuric acid	1/2,000
Histidinehydrochloride mono	1/1,000
Hydroxyproline	—
Hydroxymalonic acid	—
β-Hydroxybutyric acid	1/2,000
p-Hydroxycinnamic acid	—
p-Hydroxyphenylacetic acid	—
p-Hydroxyphenylacetic acid	1/2,000 in lanoline
p-Hydroxyphenylpropionic acid	1/2,000
p-Hydroxyphenylpropionic acid	1/2,000 in lanoline
Inositol	1/1,000
Insulinhydrochloride	1/3,000 and 1/10,000
Mannitol	1/1,000
Mannose	—
Mucic acid	—
Pyromucic acid	—
ω-Skatolylmalonic acid	—
Sperminphosphate	—
Triethanolamine	1/2,000

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EXPLANATION OF PLATES XIV TO XVI

PLATE XIV

FIG. 1. Willow shoots after eight days. (1) Ascorbic 1/100,000. (2) Control. (3) Indolylacetic 1/100,000. (4) Oestrin 1/100,000.

FIG. 2. Willow shoots after 10 days. (1) Ascorbic. (2) Control. (3) β-Indolylacetic.

PLATE XV

FIG. 3. Germination of seeds, 60 hours. (*a*) oats, (*b*) mustard, (*c*) cress. (1) Ascorbic. (2) β-Ethylindole. (3) β-Indolylpropionic. (4) β-Indolylacetic. (5) Control.

FIG. 4. Application 48 hours subsequent to germination. (*a*) oats, (*b*) mustard, (*c*) cress. (1) Control. (2) β-Indolylacetic. (3) β-Indolylpropionic. (4) β-Ethylindole. (5) β-Methylindole. (6) Ascorbic.

PLATE XVI

FIG. 5. Application 36 hours subsequent to germination. (*a*) oats, (*b*) mustard, (*c*) cress. (1) Control. (2) β-Indolylacetic. (3) β-Indolylpropionic. (4) β-Ethylindole. (5) β-Methylindole. (6) Ascorbic.

FIG. 6. Mustard grown in β-indolylpropionic 1/10,000; lying prostrate in Petri dish. Note roots.

FIG. 7. Oats grown in various concentrations of ascorbic acid: (1) 1 in 500; (2) 1 in 1,000; (3) 1 in 5,000; (4) 1 in 10,000; (5) 1 in 50,000; (6) 1 in 100,000; (7) 1 in 500,000; (8) Control.

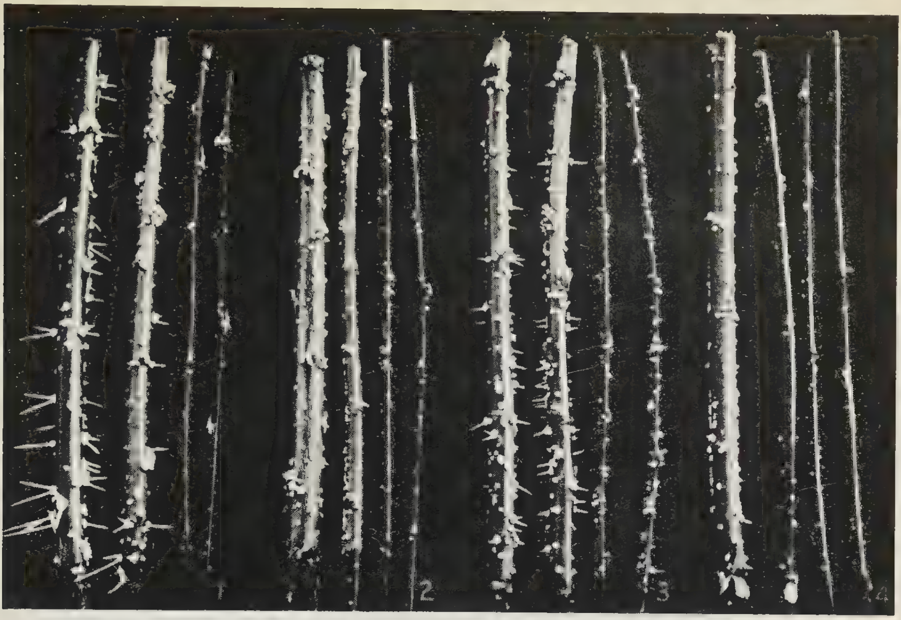

FIG. 1.

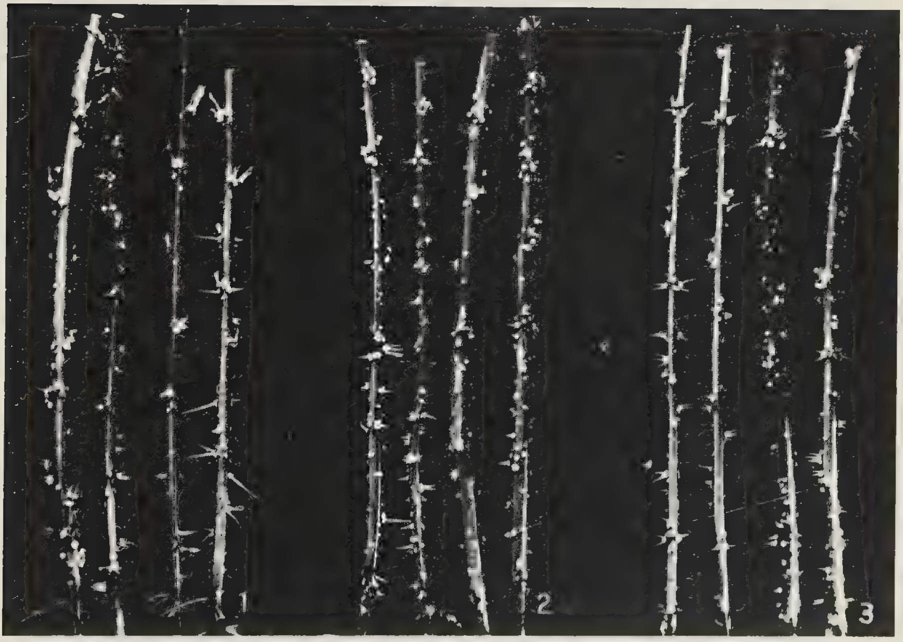

FIG. 2.

DAVIES, ATKINS, AND HUDSON – EFFECT OF PHYTOHORMONES

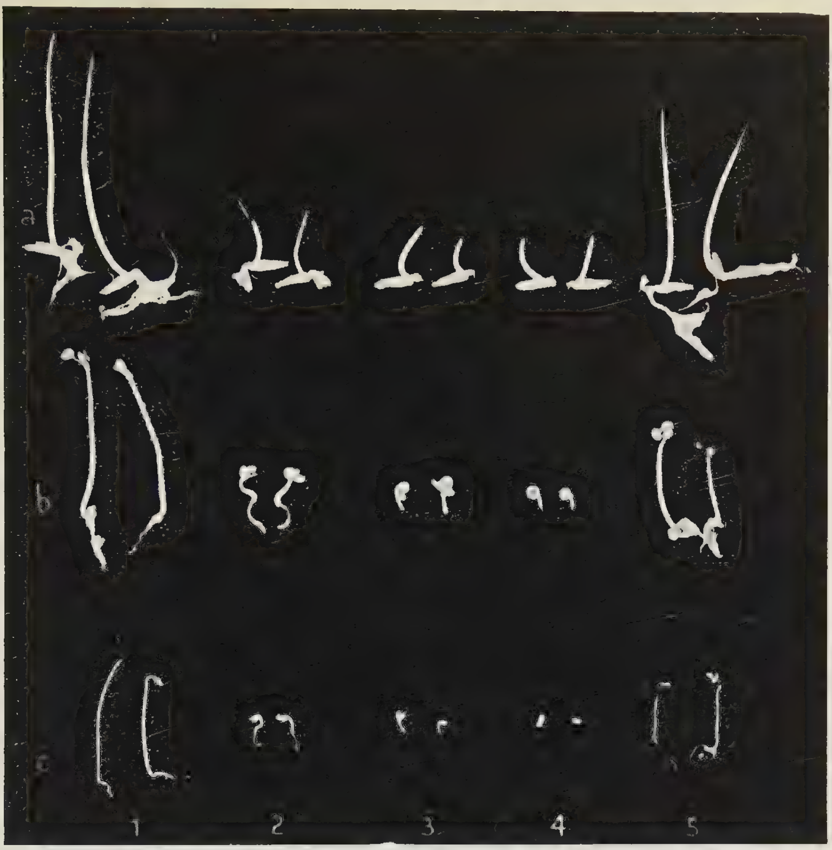

FIG. 3.

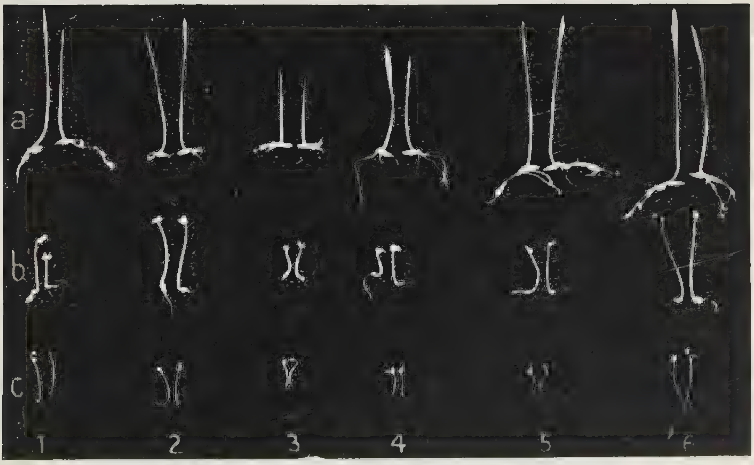

FIG. 4.

DAVIES, ATKINS, AND HUDSON – EFFECT OF PHYTOHORMONES

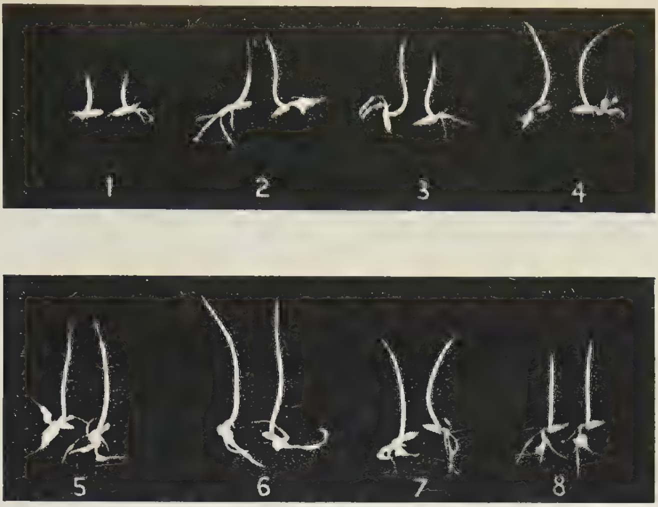

FIG. 7.

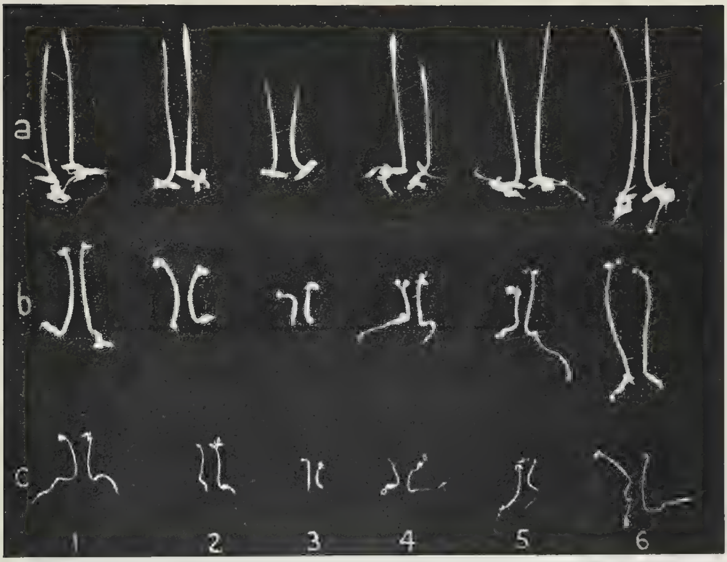

FIG. 5.

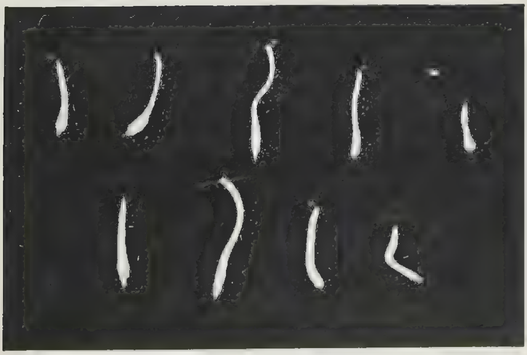

FIG. 6.

DAVIES, ATKINS, AND HUDSON – EFFECT OF PHYTOHORMONES

Studies of the Physiology of Coffea arabica

I. Photosynthesis of Coffee Leaves under Natural Conditions

BY

F. J. NUTMAN

(*East African Agricultural Research Station, Amani, Tanganyika Territory*)

With seven Figures in the Text

INTRODUCTION

IN certain of the coffee-growing areas of East Africa a serious trouble known as 'overbearing and die-back' occurs. This has been described (Nutman, 1933) and evidence presented for the view that the heavy demands for carbohydrate by the developing crop exhaust the reserves, and exceed the normal daily carbon income of the tree. The branches then die back; the greater part of the feeding roots is lost and not regenerated; and the tree is permanently injured. Although it may not die for several years it remains unproductive.

Since shortage of carbohydrate is associated with this form of die-back it is apparent that, if the rate of cropping is not to be reduced, means of control must be sought in an increase of the normal carbon income of the tree. Thus a knowledge of the rates of carbon assimilation of trees growing under plantation conditions is of importance.

This paper deals with the results of an investigation into the rates of carbon assimilation under field conditions by leaves of *Coffea arabica* growing in the coffee districts of the Northern Province of Tanganyika Territory.

METHODS

Apparatus.

Little work has been done on the march of apparent assimilation of plants in general throughout the day under natural conditions, probably because of the difficulty of devising an apparatus suitable for field use and at the same time sensitive enough to enable the rate of assimilation to be measured over a period of a few minutes. It is apparent that an apparatus depending on the absorption of carbon dioxide by an alkaline solution and the subsequent determination of the amount absorbed has a number of disadvantages, chief of these being that the result gives only the average assimilation over a considerable period of time.

The apparatus used in this work has been developed at Amani and has been used in the field with success in a travelling laboratory, even under conditions

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where sudden and violent fluctuations of temperature prevail. Improvements in certain directions might be made, but owing to the fact that the work was carried out in the tropics limitations are imposed which could not be avoided.

The apparatus is illustrated diagrammatically in Fig. 1. A large gas-holder (not shown) holds sufficient air with normal concentration of carbon dioxide to suffice for one day's work. The air passes through a sensitive gas-meter, and can then be diverted at will into one of two paths. The first is through suitable lengths of glass-tubing to a shallow elliptical cup, fastened to the under surface of the leaf under investigation, and thence to the rest of the apparatus. The second path is through a needle valve, so adjusted that its resistance to air flow is the same as that on the route to the leaf-chamber. The air can then be passed either through a U-tube filled with soda-lime or through a second needle valve adjusted to the same flow resistance as the U-tube with its connecting leads.

The air stream then passes through a conductivity cell similar to that described by Newton (1935), which proved well adapted to the work. Full details of the method of operation are given in the paper referred to and need not be quoted here. The cell holds 25 ml. of N/25 barium hydroxide solution and has a resistance of about 300 ohms at 25° C. It is kept immersed in a covered thermostat, controlled to 0·01° C. by standard methods. A lower degree of accuracy in temperature control may lead to small variations in the conductivity of the cell being masked by changes due to temperature fluctuations. The thermostat is normally kept at a temperature slightly above the maximum air temperature likely to be encountered.

The air after leaving the conductivity cell passes through a third needle valve and finally through a flow-meter of the capillary type, protected by a tube of calcium chloride from moisture which might otherwise condense inside the capillary. Suction is provided by a pair of constant-pressure aspirators.

With the exception of the lead from the gas-meter to the leaf-chamber the tubing throughout is thick-walled, glass capillary of about 1 mm. internal diameter. Hence the volume of the whole system is small, and changes in assimilation rate are reflected within a few seconds by variations in conductivity.

It will be seen that by manipulation of the stopcocks four routes are available between the gas-holder and the conductivity cell: (1) through the two needle valves; (2) over a known area of the under surface of a coffee leaf and through the second needle valve; (3) through the first needle valve and the soda-lime tube; (4) over the leaf and through the soda-lime tube. Since the resistance of the system to the passage of air is constant, irrespective of which of the four possible routes is in circuit, the rates as given by the flow-meter remain constant, and any change can readily be corrected. The flow-meter can be checked and calibrated by means of the gas-meter.

The volume of the shallow cup attached to the under side of the leaf is only 2 ml. Thus, with a rate of air-flow of 5 litres per hour the air inside the cup is completely renewed every 1·4 seconds. Boysen-Jensen and Müller (1929) suggest that the outgoing air should have at least two-thirds of the normal carbon-dioxide content, while Kostytschew, Bazyrina, and Wassilieff (1927) increase this proportion to three-quarters. In the work of the writer of the present paper this latter ratio was always exceeded.

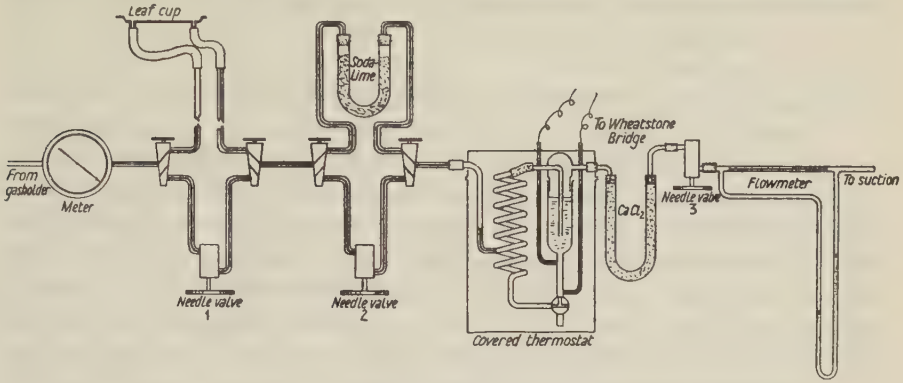

FIG. 1. Diagram of apparatus.

Heinicke and Hoffman (1933), working with apple leaves, used rates of air-flow of 2·5 litres per hour for each square cm. of leaf surface within the cup. Under these conditions the carbon-dioxide content of the air on leaving the cup was reduced by about 15 per cent. The air-supply used in the present work for coffee leaves was only 0·25 litre per hour per sq. cm. of leaf surface, that is to say, approximately one-tenth of Heinicke and Hoffman's amount. But, since the rate of assimilation of a coffee leaf averages only about 2·0 mg. $CO_2/dm.^2$/hour while the rates of apple leaves are given as reaching ten times that level, the effective rates of air-flow are similar.

The Wheatstone bridge available was of the dial type, designed for conductometric work on soils, and readings of a higher order of accuracy than 0·05 ohm could not be obtained. Alternating current for the bridge was provided by a Cambridge reed hummer, and the detection of the point of balance was by a moving coil loud-speaker preceded by a three-valve battery-operated amplifier.

Technique.

Through the courtesy of the Tanganyika Territory Department of Agriculture the author had the loan of a travelling laboratory originally designed for entomological field work. The apparatus could thus be taken alongside suitable trees growing in the plantation and photosynthetic rates of attached leaves exposed to the fluctuating conditions in the field measured.

The routine adopted in the field is as follows: The leaf is attached to the cup in the evening, and measurements made during the following day. The cup can remain attached for several days with no sign of injury to the leaf provided that an air-stream is passing throughout the day; otherwise, if the sun is shining, the temperature of the leaf rises and scorching takes place. The leaf is stuck to the flange of the cup with very thick glue. This is applied hot to the flange, allowed to cool until it is nearly set, but still 'tacky', and the leaf is then clamped lightly between the flange and a thick piece of 'sorbo' rubber for one hour.

The junction between the leaf and the cup is then tested for air-tightness by so turning the stopcocks that the air within the cup is exposed to the suction from the aspirator, while entry of air from the gas-holder is prevented. Bubbling in the conductivity cell shows that there is a leak in the system. The leaf must be darkened for some time prior to this test, since if the stomata are open the cup acts as a porometer. Entry of air into the cup through the stomata during the normal operation of the apparatus is avoided by making the lead from the gas-holder to the leaf-cup of tubing of wide bore. Since air is delivered from the gas-holder at atmospheric pressure, and the pressure difference required to operate the gas-meter is very small, the air within the leaf-cup is at a pressure so near to atmospheric that no flow through the stomata has been observed. Were any appreciable amount of air to be sucked through the stomata the readings of the gas-meter would, of course, be inaccurate.

Some hours before dawn the leaf is uncovered and the soundness of the contact between the leaf and the flange of the cup again tested. The air-stream is then started and adjusted to a suitable rate by the third needle valve; this rate is between 4 and 6 litres per hour, generally about 5 litres per hour. Air is then passed directly from the gas-meter into the cell, and the rate of change of conductivity measured. It has been found more convenient to measure the rate of change of conductivity of the cell against time than to measure the change in conductivity produced by a measured volume of air. Thus readings can be made as frequently as appears desirable. In general it was found best to take five readings at one-minute intervals, the volume of air passing for the five minutes being determined by the gas-meter. The air-stream is then passed into the cell through the leaf-chamber and the soda-lime tube. Thus no carbon dioxide passes into the conductivity cell except when measurements are actually in progress. In this way one filling of the conductivity cell will suffice for about twenty determinations, whereas if the carbon dioxide was continually passing into the cell frequent refilling and recalibration would be necessary.

The leaf is left with the air-stream passing over it until one hour or so before dawn when readings are begun. The appropriate stopcocks are turned so that the air-stream passes directly from the leaf-chamber to the conductivity cell, and, after a short interval to allow the rate of change of conductivity to become steady, five readings at one-minute intervals are taken. The leaf is then by-passed and the air-stream diverted through the two needle valves for a further five readings. This procedure is repeated at intervals, alternate determinations being made of the rate of change of conductivity with air passed direct through the conductivity cell and with the air first passed over the leaf. The difference between these rates of change corresponds to the rate of intake or output of carbon dioxide from the leaf surface attached to the chamber.

All the work reported in this paper was with horizontally placed mature leaves, the fourth or fifth leaf from the tip of a branch being selected where possible.

It would have been desirable to have taken records of meteorological conditions throughout the days on which assimilation records were obtained. This was not possible since no recording instruments were available, and the manipulation of the apparatus during periods of recording left no time for the reading of other instruments.

THE LOCALITY

The whole of this work on assimilation was carried out on the foot-hills of Kilimanjaro and Meru. An account of this region is given by Teale and Gillman (1935). Field descriptions of certain of the soils have been given in a previous paper (Nutman, 1933).

The coffee belt on these mountains extends over a varied climatic region, ranging on Meru, and to a less extent on Kilimanjaro, from the drier and sunnier northern and north-western slopes to the wetter and cloudier climate of the southern slopes.

Field work was carried out at Lyamungu, situated at an altitude of about 4,500 feet on the south-western slopes of Kilimanjaro, where the rates of photosynthesis prevailing during the dull, cloudy, and rainy weather of the 'long rains' were investigated. Later, during the drier weather, work was done on a coffee estate at Kibosho, also on the south-western slope of Kilimanjaro, and finally on an estate in the Engare Olmotonyi district of Arusha, lying to the west-south-west of Meru at an altitude of about 4,600 feet, and having a much sunnier and drier climate. Die-back was first described in this district and it is to be seen there at its worst.

THE RECORDS OBTAINED

Fig. 2 summarizes the results obtained at Lyamungu, with the addition of nine results (sun records at 7 h. to 9 h. and 15 h. to 17 h.)[1] which were obtained at Kibosho and Arusha, since during the work at Lyamungu the sun was obscured between these hours. In this figure the assimilation rate is plotted against the time of the day, circles representing results obtained when the sun was obscured and solid black dots representing those obtained when the sun was shining. In general the weather was unusually dull, as is evident from the few 'sun' determinations made during the period June–August. The 'dull' results, of course, cover a wide range of light intensities, the weather varying from slightly overcast to heavy cloud and rain. From measurements made by Mr. Moreau at Amani, light intensities range probably from less than 500 ft.-c. to over 2000 ft.-c. In general, low values of assimilation were obtained when the weather was very cloudy. It will be seen that, on the whole, there is a tendency for the 'sun' assimilation rate to be lower than the 'dull' assimilation rate during the middle hours of the day, but higher in the early morning and the late afternoon.

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[1] Times of the day throughout this paper are given in Tanganyika Standard Time, this being about half an hour ahead of apparent solar time. Times of sunrise, sunset, and local, apparent noon are inserted in the figures.

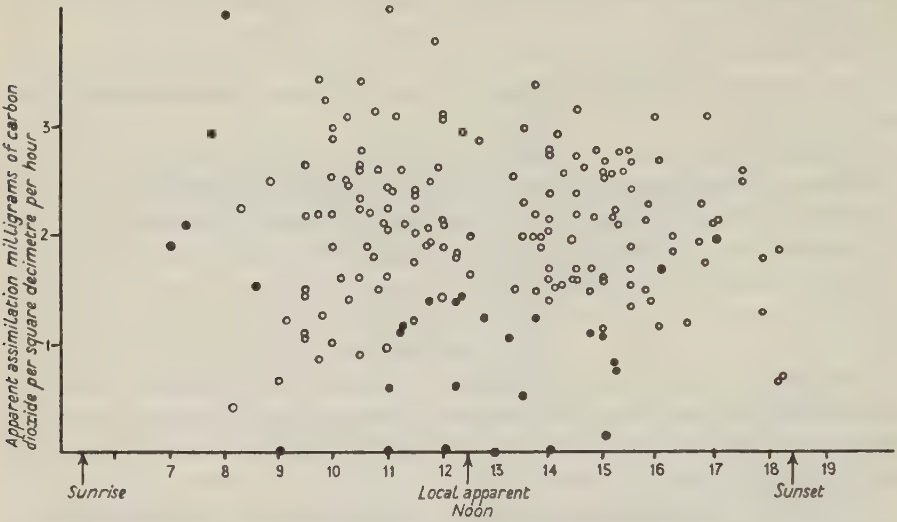

FIG. 2. Summary of 184 measurements of apparent assimilation during the months June, July, and August 1936. The weather was dull during almost the whole of this period. The results shown by solid black dots were the only ones obtained with the sun unobscured.

Fig. 3 shows the same data as Fig. 2 with the hourly means of the measured assimilation rates taken during successive hours plotted against the time of day, the dotted line representing determinations made during cloudy periods, and the unbroken line those made in sunshine. Above each point on the graph the number of determinations averaged is shown. The curve for the 'sunny' periods is less accurate than the other as the observations are fewer and no allowance is made for a possible cumulative effect of insolation. Even so, the implications of the graph are obvious. During cloudy weather the assimilation remains at a fairly constant value throughout the day. In the sun, on the other hand, the rates show a large depression during the midday hours. These results, derived from two dozen trees and more than 100 leaves over a period of several months, confirm, in general deductions made from more detailed studies of the diurnal variation in assimilation rates of single leaves.

The results which follow (Figs. 4–7) are from studies of the rates of photosynthesis of single attached leaves throughout the day. Each graph is selected as being representative of the behaviour of the leaf under the conditions stated, and a sufficient number of determinations at each station have been carried out to show that they are typical.

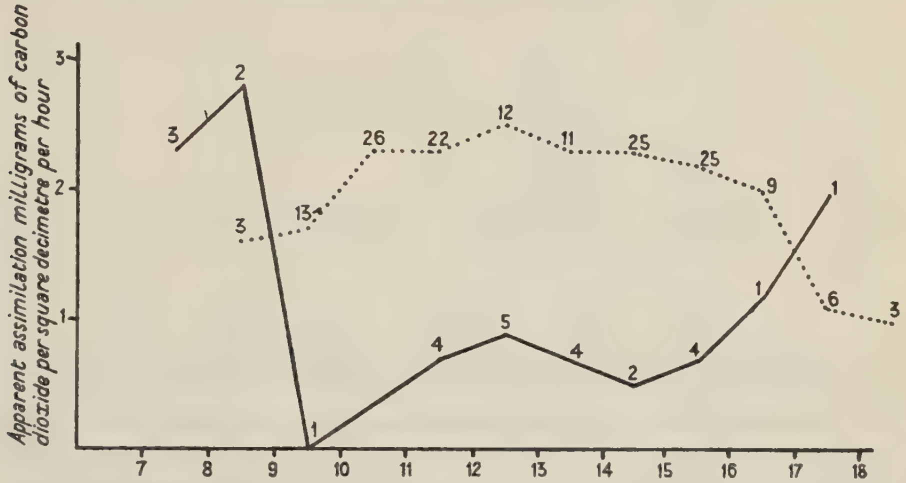

FIG. 3. For full details see text. Assimilation rates of *C. arabica* (dotted line) during cloudy periods and during sunny periods (unbroken line). The figure above the line is the total number of observations averaged for each point on the curve.

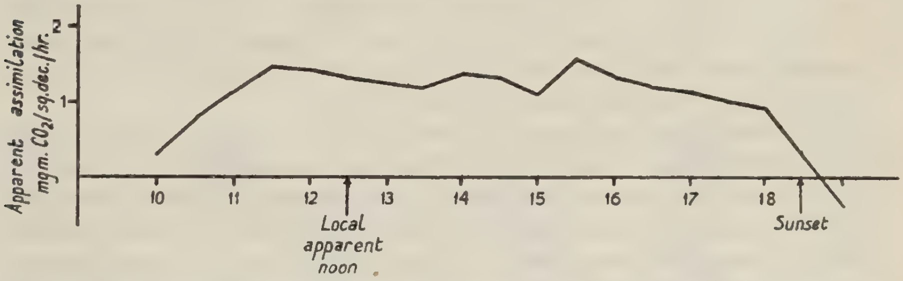

FIG. 4. Lyamungu. Apparent assimilation rates of a leaf fully exposed to an exceptionally dull, cold, and overcast day during the 'long rains'. Rain was falling during most of the time.

Fig. 4 represents the march of apparent assimilation of a single unshaded leaf at Lyamungu during extremely dull weather on August 26, when rain was falling during the whole day excepting from 15 h. to 16 h. The low value of assimilation is noteworthy as is the fact that there is no drop in assimilation rate during the midday hours.

Fig. 5, obtained on September 18 is from an estate at Kibosho shaded by *Grevillea robusta*. The shade provided is denser than normal and is probably denser than the optimum for coffee. Since *Grevillea* is a tree of upright habit it was not found possible to select a coffee tree in uniform shade. The leaf selected was unshaded from 7 h. 15 m. to 8 h. 30 m., from 10 h. 25 m. to 10 h. 45 m. and from 11 h. 25 m. to 12 h. 05 m. During the rest of the day it was in deep shade. On the day during which these results were obtained morning mist persisted until 9 h. 15 m. Assimilation began at about dawn, and slowly rose to 2·6 mg. CO_2/dm.^{2}/hour at 10 h. Soon afterwards bright sunlight fell on the leaf, and the assimilation at 10 h. was zero. At 11 h. the leaf was in light shade, the assimilation rising, although not to so high a value as that recorded in full shade. At 11 h. 30 m. when the leaf was again in the sun the assimilation had fallen again to zero. When the leaf came under shade at 12 h. 05 m. assimilation rose, reaching 3 mg. CO_2/dm.^{2}/hour at 13 h. A rate of over 2 mg. CO_2/dm.^{2}/hour was maintained until 16 h., when a fall began and at sunset the apparent assimilation was about zero. Respiration became apparent shortly afterwards at a value of approximately 0·75 mg. CO_2/dm.^{2}/hour which was still maintained at 21 h.

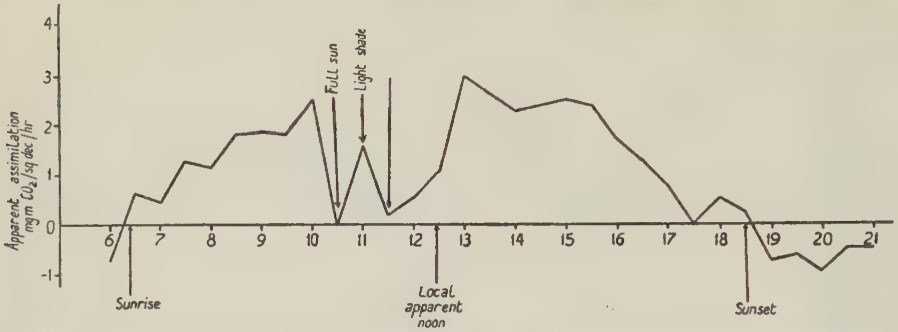

FIG. 5. Kibosho. Rates of apparent assimilation of a coffee leaf throughout the day under heavy *Grevillea robusta* shade. From 10 to 12.30 the shade on this leaf was very slight.

Fig. 6, obtained at Arusha on October 1, is a curve from an estate lightly shaded with *Grevillea robusta*. The varying degree of shade to which the single leaf was exposed is indicated in the diagram. 'Very light shade' means that the leaf was screened by a high branch of *Grevillea* and thus exposed to sun-flecks and shade of the penumbra type. 'Light shade' is shade of the penumbra type but with few sun-flecks. During the two days represented on this graph the sky was completely cloudless. The temperature was somewhat higher than was experienced at Kibosho. Assimilation began as before at about dawn but rose more rapidly than at Kibosho, probably as a response to the clear bright morning. At 8 h. the assimilation was higher than that recorded at any time at Kibosho. At 9 h. the leaf, still in full sun, showed the beginning of a fall in assimilation rate, which was maintained and increased somewhat during the 'very light shade' period. By 12 h. the assimilation had fallen to 1·5 mg. CO_2/dm.^{2}/hour, but rose rapidly to a maximum of 4·3 mg. CO_2/dm.^{2}/hour at 13 h. as a response to the shade during the previous hour. When this patch of shade was replaced by 'light shade' assimilation again fell to less than 2 mg. CO_2/dm.^{2}/hour at 14 h., while it

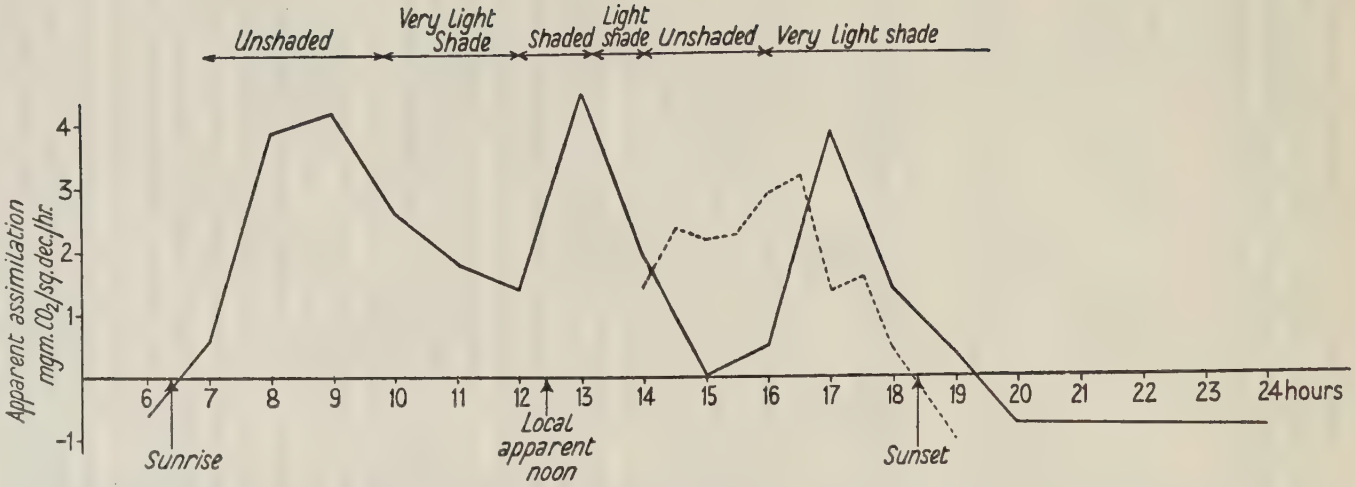

Fig. 6. Rates of apparent assimilation of a coffee leaf throughout a cloudless day from an estate shaded by *Grevillea robusta*. The dotted line is the apparent assimilation of the same leaf for part of the following day, but with an opaque screen placed one foot above it.

approximated to zero during the sunny period from 14 h. to 16 h. A rise occurred prior to the evening fall, although the leaf was only in light shade, since the sun's rays were now falling obliquely on the horizontal leaf.

The dotted line on the curve represents readings obtained on the same leaf during the following day, October 2. The assimilation rates during the early part of the day were similar to those for the previous day and are omitted. At 14 h., when the fall in assimilation rates accompanying the increase of light intensity at 13 h. was taking place, the leaf was shaded by a piece of cardboard one foot square placed one foot above the leaf. Assimilation at once increased. The rates attained were not quite so high as those of the previous day, but the shade applied was so dense that besides all direct light from the sun, the radiation from a considerable portion of the sky was also shut out.

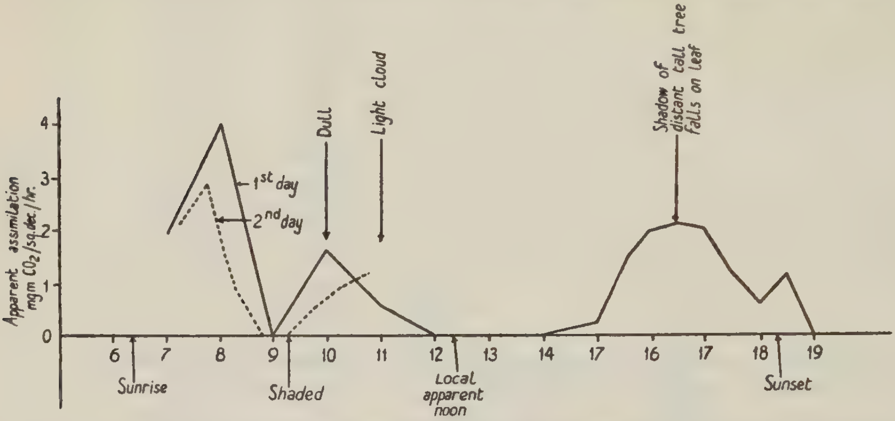

FIG. 7. Arusha. Rates of apparent assimilation of a leaf exposed to the full sun throughout the day. Clouds obscured the sun at 10 and 11. The dotted line shows the rates of the same leaf on the following day, which was cloudless from 7.15 till noon. At 9.15 the leaf was shaded by one thickness of cambric.

Fig. 7 shows the march of assimilation rates on October 9 from an unshaded tree on the same estate as in Fig. 6. Unfortunately no more completely cloudless days occurred, although on this day the sun was unobscured except for a dull period at 10 h. and a little light cloud at 11 h.

Assimilation rose rapidly after sunrise, reaching 4 mg. CO_2/dm.^{2}/hour by 8 h., but falling to zero at 9 h. Thus this leaf ceased assimilation rather earlier than that shown in Fig. 6. This is probably due to the fact that by 9 h. this leaf was exposed both to the direct rays of the sun and also to the radiation from almost the whole of the sky, whereas at the same hour the leaf in Fig. 6, while exposed to the sun, was screened from almost the whole of the sky radiation. It will be observed that at both 10 h. and 11 h. when the sun was obscured assimilation was appreciable. In none of the records obtained during these hours when the sun was shining was there any measurable assimilation. During the late afternoon, when the sun's rays were falling obliquely on the leaf, assimilation again set in, but fell to zero soon after sunset.

The dotted lines on the chart represent the assimilation during the following day, October 10, which was cloudless in the morning. After the initial rise in assimilation rate, as on the previous day, assimilation fell to zero at 8 h. 45 m., remaining at that level until 9 h. 15 m. The leaf was then screened with one thickness of unbleached linen. Assimilation at once began again and the rate was rising at 10 h. 45 m. when the experiment was discontinued.

Summary of results.

From the data presented above the following table has been prepared.

TABLE I

Apparent Assimilation (mg. CO_2/dm.^{2}/hour) of Attached Coffee Leaves under Field Conditions

Maximum rate	4·5
Average value from 8 h. to 19 h. of unshaded leaves under dull conditions	1·9
Average value for all daylight hours of unshaded leaves on a very dull day	0·9
Average value for all daylight hours on a heavily shaded estate on a normally bright day	1·4
Average value for all daylight hours on a moderately shaded estate on a cloudless day	2·1
Average value for all daylight hours on an unshaded estate on a cloudless day	0·7

It is of interest to compare these results with assimilation rates as published for other plants. The following table summarizes some fairly recent work.

TABLE II

Apparent Assimilation (mg. CO_2/dm.^{2}/hour) of Leaves Exposed to Normal Concentration of Carbon Dioxide

Plant.	Observer.	Apparent assimilation.	Remarks.
Potato	Lundegårdh (1931)	9·57	Assimilation stated to be measured under conditions of optimum light and normal carbon-dioxide concentration.
Tomato	”	8·42	
Sugar beet	”	9·26	
Spinach	”	9·78	
Vicia faba	Walther	8·83	
Phaseolus vulgaris	Yoshii	9·27	
Oxalis acetocella	Lundegårdh (1931)	2·4	
Cow pea	Miller (1931)	18·0	
Apple	Heinicke and Hoffman (1933)	15·0	Assimilation was measured under natural illumination in the summer. Maximum value over a 5-hour period was over 20.
Geranium	Mitchell	16·5	Illumination by arc light. Carbon-dioxide concentration is ‘within the range of variability under natural conditions’.
Tomato	”	17·0	
Primrose	”	13·5	
Cineraria	”	13·5	
Wax bean	”	13·0	

TABLE II (*contd.*)

Plant.	Observer.	Apparent assimilation.	Remarks.
Musa textilis			Illumination — tropical sunlight. Carbon dioxide was from normal air, but carbon-dioxide content of leaf-chamber was considerably reduced by assimilation, hence the effective concentration was sub-normal.
average value .	McLean (1920)	0·42	
maximum ,, .	,,	1·03	
Cocos nucifera			
average value .	,,	0·39	
maximum ,, .	,,	0·67	
Sugar cane			
average value .	,,	3·6	
maximum ,, . .	,,	5·0	

A very considerable range of variation is to be seen in these results, but, with the exception of Oxalis, an extreme shade plant, they fall into two distinct groups, typical tropical sun plants and others, the former showing much lower assimilation rates, though one might expect high assimilation rates under tropical conditions; Lundegårdh (1931) goes so far as to say 'the efficiency of the leaves in the tropics is much greater than those of temperate plants'.

The figures obtained for coffee are also low, but they may be the low rates typical of a sciophyte rather than those general for tropical plants. It is regrettable that more data are not available on the assimilation rates of tropical plants under natural conditions.

Additional observations.

During the course of this work a number of additional observations were made. The results of these are summarized below.

1. Additional water-supply to the roots of the tree does not affect the main features of the march of assimilation rates; in particular the midday closure in the sun is unaffected.

2. When all the leaves of the tree except the experimental leaf are covered by an opaque cloth so that they are in almost complete darkness and in still air, the behaviour of the experimental leaf is in no way different from that observed when the other leaves—these may be as many as 5,000—are exposed.

3. The effects of shade on the assimilation of a leaf is the same whether that leaf alone is shaded or shade is applied to the whole tree.

4. A horizontal leaf exposed to the midday sun and assimilating at a rate nearly zero will at once increase its assimilation if turned edgeways to the incident light.

5. A horizontal leaf, exposed to full sunlight for 16 hours, and assimilating at an appreciable and increasing rate, if turned so as to be normal to the incident light will show decreased assimilation.

7. Assimilation rates can change very rapidly. The following table illustrates this well and summarizes two-minute determinations of photosynthetic

rates on July 13 when cloud and sunshine were rapidly alternating. Had the readings been taken at irregular intervals, corresponding to the light fluctuations, the results would doubtless have been even more striking. The last three readings were taken with the leaf under investigation shaded from the sun by a small piece of cardboard, the remainder of the tree being in full sun.

TABLE III

Photosynthetic Rates of Attached Coffee Leaves over Successive Two-minute Intervals, July 13

Weather.	Apparent assimilation mg. CO_2/dm.^{2}/hour.
Dull . . .	2·4
,, . . .	1·8
Sun . . .	1·2
,, . . .	0·6
,, . . .	0·6
Dull . . .	1·2
,, . . .	2·4
Sun . . .	1·2
,, . . .	0·6
Variable . .	0·6
Sun (shaded) .	2·4
,, ,, . .	2·4
,, ,, . .	2·4

DISCUSSION

Perhaps the most interesting feature of the daily march of assimilation rates is the midday drop well shown in Fig. 7. This has been noted by other workers, and was observed in tropical crops by McLean (1920), who suggested no reason for it. Leaving on one side the possibility that some unspecified change in the internal condition of the leaf is responsible, which is merely restating the problem, and the suggestion that solarization has taken place, which is negatived by the rapidity of recovery, three hypotheses suggest themselves for serious consideration. These are:

1. That the midday drop is due to a reduction in the water content of the leaf.
2. That it is due to the excess of accumulation of assimilates.
3. That it is due to stomatal closure.

These hypotheses will be considered in turn.

1. *The 'Water-content' hypothesis.*

It has been shown by a number of workers that assimilation is slowed down when the water content of the leaf is reduced. For tropical plants, Dastur and Desai (1933) have shown that a correlation exists between the water content of the leaf and its photosynthetic activity. This hypothesis does not appear to be applicable for the following reasons. (1) It is probable that the water relations of a single leaf would be conditioned by that of the whole tree, and hence treatment applied to the single leaf would be ineffective in restoring its water content, but as we have seen, each leaf behaves independently. (2) Covering all the leaves of a plant might be presumed to increase the water-supply to the single exposed leaf since the stomata would probably close in the darkness, and in any case the covering would certainly reduce transpiration very considerably. No change, however, in the reaction to sunlight of the single exposed leaf has been observed. (3) Turning a leaf so as to alter its relation to the incident light immediately alters its assimilation rate, though such a procedure cannot immediately alter its water content. (4) Copious watering of the plant has no effect. (5) Table III shows clearly how quickly light fluctuations are followed by changes in photosynthetic rates. It is improbable that the water content of leaves can change so rapidly. (6) The young leaves of coffee are somewhat sensitive to water-strain, and show signs of wilting long before the rest of the plant is affected. No such signs were ever observed on any experimental tree.

2. *The hypothesis of accumulation of assimilates.*

Saposchnikoff and Ewart, as quoted by Spoehr (1926), have shown that accumulation of assimilates in the leaf when assimilation exceeds translocation can exert an inhibitory action on photosynthesis. Such an explanation is not applicable to the midday fall for the following reasons: (1) Table I shows that the total daily assimilation of a shade leaf which shows no midday fall in assimilation is three times as great as that of a leaf in the sun which shows a marked midday fall. If the products of photosynthesis accumulate in the sun leaf to such excess that assimilation is inhibited, the shade leaf with a higher photosynthetic rate should accumulate even more, unless a subsidiary hypothesis of accelerated translocation in the shade leaf is introduced. (2) The shading of a leaf assimilating at zero rate and hence, by hypothesis, choked with assimilates suffices to bring about an immediate increase in assimilation, as does the turning of the leaf edgeways to the incident light. It is impossible to hold that such treatments bring about immediate reduction of the contained assimilates. (3) Conversely, the observed fact of the inhibition of photosynthesis when a leaf is turned normally to the incident light during the late afternoon is inexplicable on this hypothesis.

3. *The hypothesis of 'stomatal closure'.*

The stomata are the main paths of gaseous exchange, so the results might be explained by variation in the rate of ingress of carbon dioxide to the leaf due to stomatal movement. Were these movements influenced by light in such a manner that the stomatal aperture is directly correlated with light intensity at low light intensities, and inversely correlated when light intensities are high, the observed phenomena would be adequately explained. In a subsequent paper evidence will be presented to show that the stomatal movements of coffee are of the type required by this hypothesis.

SUMMARY

1. A method is described whereby the rates of uptake of carbon dioxide by attached leaves of *Coffea arabica* can be measured over short intervals of time.

2. Data are presented for the apparent assimilation of leaves of *Coffea arabica* under field conditions, and the diurnal march of assimilation rates is determined for a number of habitats.

3. Assimilation rate appears to vary directly with light intensity when this is low, but high light intensity reduces the rate. Under all conditions of light the time-lag between a change in light intensity and a change in assimilation rate is less than two minutes.

4. The assimilation rate of a coffee tree is greater under conditions of moderate light intensity than in full sunshine, and the total daily assimilation is greater in the shade than in the sun.

5. Evidence is presented as to the cause of the midday drop in assimilation rate during periods of sunshine, and the conclusion is reached that neither the water relations of the leaf nor the possible accumulation of assimilates can explain this phenomenon.

ACKNOWLEDGEMENTS

The writer gratefully acknowledges the loan of a travelling laboratory by the Tanganyika Department of Agriculture; without it this work could not have been done. His thanks are also due to the Chief Scientific Officer of the Coffee Experimental Station at Lyamungu, for providing facilities for work there; and to Mr. W. H. Baldwin, and Mr. F. A. F. Schmid, for permission to work on their estates.

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Chromosome Studies in Hyacinthus orientalis L.

III. Reversal of Sexual State in the Anthers of Hyacinthus orientalis L., var. Yellow Hammer

BY

S. P. NAITHANI

With Plate XVII

INTRODUCTION

WHILST studying the meiotic divisions in the different varieties of *Hyacinthus orientalis*, the author came across giant pollen grains in the variety *Yellow Hammer*, which showed striking resemblances to embryo-sacs. Němec, as far back as 1898, was the first to report such embryo-sac-like pollen grains in the partial petaloid anthers of *Hyacinthus orientalis* and to describe their development. De Mol (1921) observed this 'Němec's phenomenon' in a number of varieties. Stow (1930) obtained similar giant pollen grains in the normal anthers of the variety *La Victoire* by submitting the bulbs to high temperature during the reduction division of the pollen mother-cells. He (1933) concluded that these abnormal pollen grains possessed female tendencies. In the present case the embryo-sac-like pollen grains were found in the normal anthers of the variety *Yellow Hammer*.

MATERIAL AND METHODS

Bulbs of different varieties of *Hyacinthus orientalis* specially prepared for early flowering were used. M. van Waveren & Sons, Holland, suppliers of bulbs to Messrs Alexander & Brown of Perth, from whom the bulbs were obtained, had applied the following special treatment. Bulbs were lifted about the middle of June when they were still in full growth. The usual time of ripening off being about the middle of July, hyacinths destined for preparations still had green foliage at the time of lifting. The bulbs were then spread out in the warehouse in trays or on racks and the warehouse was moderately heated during the first few days to a temperature of about 70° F. After about five days the temperature was raised to about 80° F., keeping the windows closed, the bulbs thus lying in the dark. Care was taken to keep the atmosphere of the warehouse slightly moist, while sufficient ventilation was applied in order to remove all foul air. The bulbs were then left in a temperature from 80° to 82° F. for five weeks, after which the temperature was gradually lowered to about 70°. On July 25 the preparing process was finished and the bulbs were exported.

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After receiving the bulbs from Perth they were kept in the Botanical Laboratory, King's College, at room temperature (60° F.).

The bulbs were dissected and the anthers examined from time to time. Pollen grains were first examined in Belling's iron aceto-carmine. Anthers were then smeared, fixed in La Cour's 2 BE and stained by Newton's iodine gentian-violet technique.

OBSERVATIONS

At the end of the month of October 1935 the anthers of the variety *Yellow Hammer* showed embryo-sac-like giant pollen grains. The anthers of other varieties examined, though similarly prepared for early flowering, possessed normal pollen grains only.

The development and the formation of these giant pollen grains resembled exactly that of the embryo-sac. After the tetrad division, the young pollen grain grows and becomes larger than the normal (Pl. XVII, Fig. 1). It contains a large quantity of food material. Fig. 2 represents a normal pollen grain of similar age. A comparison of the two shows an appreciable size difference. These pollen grains are accompanied by abortive pollen grains (Fig. 3) amounting to about 60 per cent. of the whole. Stow found more than 90 per cent. dead pollen grains in the variety *La Victoire*.

In the development of the abnormal pollen grains the extine breaks while the pollen is still uninucleate and the intine protrudes and swells up to form a bulbous structure (Fig. 4). The nucleus then divides into two (Figs. 5, 6). This division takes place in the middle of the short tube and then the daughter nuclei migrate to opposite ends of the sac. Figs. 7 and 8 represent the metaphase and anaphase of the first division respectively. In some cases the nucleus divides before the formation of the tube (Fig. 9). The tube now elongates and the nucleus at each end divides into two, forming a four-nucleate structure (Fig. 10). Fig. 11 illustrates the metaphase of the second division. In some cases the two nuclei, before passing to opposite ends of the sac, divide, producing four nuclei at the middle of the tube (Fig. 12). After the third division the eight-nucleate embryo-sac-like structure is formed showing four nuclei at each pole (Fig. 14). Fig. 13 represents the prophase of the third division. The tube by now has elongated considerably and has become more bulbous. One nucleus from each end migrates to the centre, where the two then lie close together. All of the nuclei now become surrounded with a definite cell-wall. One of the cells towards the extine or pollen grain end becomes larger and forms the egg-like cell, the other two corresponding to synergids. The three at the opposite pole resemble the antipodals and the two in the centre the polar nuclei (Fig. 15). Thus the whole structure resembles in every respect a typical mature embryo-sac, of course with the difference that (1) it is not surrounded by the nucellus and integument but lies free in the pollen-sac, and (2) it has extine towards the end at which the egg is situated. Stow

(1930) found that the polar nuclei fused, but I did not observe the fusion of the polar nuclei.

Variations from the above type of structure were observed. In one case the egg-apparatus consisted of the 'egg cell' only, the other seven being scattered in groups of one, two, and three each (Pl. XVII, Fig. 16). Figs. 17, 18, show further haphazard arrangement of the nuclei, some of which have fused.

DISCUSSION

Němec (1898) stated that in the development of the abnormal pollen grains in the partial petaloid anthers the generative nucleus degenerated and the vegetative nucleus through three successive divisions formed this structure. He further supposed that the deviations were the consequence of anthers being petaloid. De Mol (1921), from his experimental results, came to the conclusion that only anthers of those bulbs which were dug up in an unripe condition between June 1 and 15 and then immediately artificially heated until September 20 showed pollen embryo-sacs. He further (1923) describes types of pollen grains which appear to be stages in the development of pollen embryo-sacs. He considers this phenomenon to be the duplication of generative nuclei by means of 'physiological stimuli'.

Stow (1930), from his experimental results on the variety *La Victoire*, observed that pollen-embryo-sacs are produced only if the bulbs are subjected to high temperature (20° C.) at the time of meiosis in the pollen mother-cells and they are further forced in the greenhouse. He further observed that the abnormal pollen grains are always accompanied by dead pollen grains amounting to more than 90 per cent. of the total. He (1933) determined the relative reduction power and hydrogen-ion concentration in the normal and in the abnormal pollen grains by means of colour indicators. He found that the intramolecular reduction power of the cytoplasm of the pollen embryo-sac was greater than that of the normal grains. Stow observed that when pollen embryo-sacs together with some normal pollen grains of another garden variety were cultured on an agar-medium, the pollen tube of the normal variety grew towards the pollen embryo-sacs and entwined round them. In one case a sperm nucleus out of the tip of a pollen tube was observed just penetrating the embryo-sac-like pollen grains. In another case he found that the polar nucleus was divided into sixteen nuclei and supposed that this had resulted from the fusion of the sperm nucleus with the polar nucleus. But he could not obtain any definite evidence of the fusion of a sperm nucleus with the egg nucleus nor of the formation of an embryo. Stow's investigation clearly indicates that these embryo-sac-like pollen grains possess female tendencies not only in development and morphological structure but also in physico-chemical characters of the cytoplasm, suggesting thereby that a reversal of sex has taken place in them. They are not due to the duplication of the generative nuclei as De Mol supposed.

Regarding their production, Stow assumed that the dead pollen grains produce 'necrohormone' which causes the nuclei of the surviving pollen grains to divide. The action of such a 'hormone' he considers to be closely linked with the stage of development of the pollen grains and the temperature. He further assumes that the male nucleus of the young pollen grain has tendencies to develop into either the male or the female form. In normal conditions the 'male potency' is dominant over the 'female' so that the generative nucleus divides to form male nuclei. But in abnormal conditions, when an abundance of 'necrohormone' produced from the dead pollen grains acts together with a suitable temperature, the dominancy of the 'male potency' is weakened or disappears, so that the 'female potency' becomes stronger and therefore the embryo-sac-like structure is formed.

From my observations and those of De Mol it appears that the three conditions mentioned by Stow are not the only ones capable of producing embryo-sac-like pollen grains. The special treatment given to the bulbs at the time of their lifting in an unripe condition produces the necessary changes in *Yellow Hammer*. The percentage of abortive pollen grains has no significance; it may vary with the variety.

The production of the abortive pollen grains is probably not the cause, as the 'necrohormone' conception of Stow demands, but the consequence of these embryo-sac-like structures. Since the food material is limited in the pollen-sac, only a few pollen grains succeed in developing while others abort.

The investigation of this question leads us to the problem of the determination of sexual differentiation. In recent years there have been two rival theories in the field concerning sex and its determination. They are known as the Mendelian and the metabolic theories. According to the Mendelian theory sex, like other characters of organisms, is regulated by internal factors known as genes. This view is supported by the fact that in many animals males and females differ visibly in their chromosome complement. In recent years considerable strength has been given to this conception by the discovery of sex chromosomes in certain plants. The first sex chromosomes in plants were discovered by Allen (1917, 1919) in the liverwort *Sphaerocarpos Donnellii*, where he found an unequal XY pair. This was followed by Santos's (1923, 1924) discovery of an unequal XY chromosome pair in *Elodea canadensis*, the first known case of sex chromosomes among the monocotyledons. At the same time Winge (1923), and Miss Blackburn (1923) described independently sex chromosomes in *Melandrium album*. Both investigators reported an unequal XY pair in the staminate plants. Miss Blackburn found an equal pair in the pistillate plant. This is the first recorded instance of sex chromosomes in dicotyledons. These were followed by other cases.

The metabolic theory regards sex as a purely physiological condition influenced by environmental factors. This view, although admitting the validity of the Mendelian theory for other characters, holds that the phenomena of sexuality have no connexion with heredity units. This conception is largely the outgrowth of much experimental evidence of sex expression in plants being controlled by environmental factors. Nagai (1915) observed that the development of sex organs in homosporous fern prothallia could be controlled by varying cultural methods. Camp (1929) showed that male tissue has relatively greater catalase activity than female. Tally (1934) found that in hemp the staminate plants have a higher average percentage of total carbohydrates, polysaccharides, and sugars than the pistillate plants and that nitrogen is relatively more abundant in pistillate plants than in the staminate plants. Further support was given to this view by the fact that sex reversals in plants can be brought about by appropriate alterations in the environmental conditions. The work of Schaffner (1917, 1921*a*, *b*, 1922, 1923, 1929, 1930) in this field is well known. By varying the light intensity, photoperiodicity, moisture conditions, nutrition, and other factors, he has been able to bring about complete reversal of sex in *Cannabis sativa*, *Morus alba*, *Humulus japonica*, and various other dioecious plants. From his experimental results he comes to the conclusion that sex in plants is a purely physiological condition having no relation whatsoever with sex factors.

The Mendelian theory does not deny the influence of external factors. The modern theory of heredity attempts to state in simple formulae the observed behaviour of the inherent capacities of an organism and to explain the strong disposition of the individuals to develop certain characters under certain conditions. What are inherited are merely the abilities to react in certain ways under certain conditions. The conception of an interacting system embracing the two sets of factors, the 'intrinsic' and 'extrinsic', has been effectively discussed by Emerson (1923) and Sharp (1924).

There is no dearth of instances where environmental conditions have been observed to influence the expression of vegetative characters whose Mendelian inheritance is well known. To cite one typical case, it has been observed that in some strains of maize a red pigment (sunred pericarp) develops in the pericarp if the ear is exposed to light at the proper time, and no such colour is produced if the ear is not so exposed.

Moreover, sex inheritance is not so simple an affair as has been supposed. Sex is probably an expression of the interaction of several, perhaps many, factors located in different chromosomes. In maize, at least nine pairs of genetic factors have been found which influence the expression of sex. It is not yet definitely known whether these are all located in different chromosomes, but several of them have been found to be inherited independently. Sex is therefore as much under the control of Mendelian factors as is any other vegetative character.

According to the prevailing view sex is determined fundamentally by the sum of the hereditary units pulling in female or male direction. If the genes that lead the development in a female direction are preponderant the individual will develop into a female. If the male genes are preponderant the individual will be a male. But if the two sides are near balance intersex forms may make their appearance. It has been definitely established that sex chromosomes possess genes which are strong sex-determinating factors. Therefore under normal conditions they act as decisive sex determinants. Further, it has also been demonstrated that autosomes contain sex genes, masculine as well as feminine, and they sometimes assert their influence in the sex determination. Winge (1934), by his experimental studies on *Lebistes reticulatus*, has demonstrated that the difference between the sex chromosomes and the autosomes is essentially quantitative in nature so far as the power of sex determination is concerned. He, by crossing and back-crossing experiments, was successful in altering a pair of autosomes into sex chromosomes, in the systematically produced race in which females as well as males have XX. In the same way he was able to alter X-chromosomes into autosomes, so that X-linked genes are inherited in ordinary autosomal Mendelian manner.

Grave and Smith (1936) observe inversion of sexes throughout the life of the shipworm, *Toredo navalis*. They believe this inversion to be due to genetic causes rather than to environmental. Pincher (1935) has recently given a genetical interpretation to the origin of heterospory and allied phenomena of sex.

In the light of the modern Mendelian theory then, the condition in *Yellow Hammer* can be explained as follows: Factors influencing maleness and femaleness are present in all the chromosomes of the microspores. Under normal conditions the balance is more strongly towards maleness and so a male gametophyte is produced. But with a change in the environment the balance is changed towards femaleness, and so a female gametophyte is produced. The fact that sex reversal occurs in the variety *Yellow Hammer* only and not in other varieties, though they were also similarly treated, gives additional support to the genetical interpretation of this condition. In the other varieties, the genetic balance is such that the environmental condition which disturbs the balance in *Yellow Hammer* is not effective. The same can be said of many other plants, that in them the genetic balance is so strong towards maleness or femaleness that the reaction is undisturbed in any environmental conditions yet tried. It is possible that the pollen of many other plants might be induced to form a female gametophyte by subjecting it to an environment quite different from that which it normally encounters.

The physiological conception based by Schaffner on his experimental results cannot be regarded as critical from the genetical view-point. It lacks breeding tests. According to his metabolic theory, if the plants whose sexes have been reversed are crossed among themselves or with normal ones they should always give 1 : 1 ratio. But if abnormal ratios are obtained, his theory fails.

Let us consider two hypothetical instances in plants from the Mendelian point of view, where XX represents the female and XY the male.

I. Where male reversed in expression into female is crossed with a normal male.

XY (rev. into ♀) × XY (normal ♂), we get 1 XX : 2 XY : 1 YY (super male). A similar type of ratio was obtained by Huxley (1920) in the fish Girardinus, when a normal male (XY) was mated with a 'somatic' (XY) female.

II. Where the female reversed in expression to male is crossed with a normal female.

XX (normal ♀) × XX (rev. into ♂), XX all females are obtained.

This type is well illustrated by an abnormal reproductive system in the frog. Crew (1921) observed in the frog that an individual which at one time possessed solely the complete sex-equipment of the female comes to exhibit the organization of the male. Such a transformed individual not only has the male organization but also behaves and functions as a male. When such a male fertilized the eggs of a normal female only females were produced. Crew (1921) and Huxley (1922) explain this behaviour on the conception that the frog has a chromosome constitution of the XY (♂), XX (♀) type. Since all the gametes of the normal female and the female transformed into a male which fertilized it would contain an X, all the offspring would therefore be XX—i.e. female.

Similar breeding experiments in plants, especially those whose sexes are reversible by environmental conditions, would throw a great deal of light on the problem of sex in plants.

SUMMARY

In the anthers from bulbs of *Hyacinthus orientalis* variety *Yellow Hammer* specially treated by breeders for early flowering, the microspore nuclei undergo three successive divisions, producing giant pollen grains whose gametophyte closely resembles the typical embryo-sac in Angiosperms.

Other varieties similarly treated and also those untreated possessed only normal pollen grains.

The special temperature treatment given to these bulbs at the time of lifting in unripe condition is shown to be the cause for the production of such structures.

This reversal in the sexual state of the pollen grains is interpreted in the light of modern Mendelian theory of sex determination.

The genetic balance in other varieties is such that the temperature treatment which upsets the balance in *Yellow Hammer* is not effective in altering the development of the pollen grains in those varieties.

I wish to express my gratitude to Professor R. Ruggles Gates, for his invaluable help and suggestions during the course of this investigation.

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EXPLANATION OF PLATE XVII

Illustrating Dr. S. P. Naithani's paper on 'Chromosome Studies in *Hyacinthus orientalis* L. III. Reversal of Sexual State in the Anthers of *Hyacinthus orientalis* L., var. *Yellow Hammer*'

All figures were drawn from var. *Yellow Hammer* with the aid of Reichert camera lucida at table level. Magnification 500

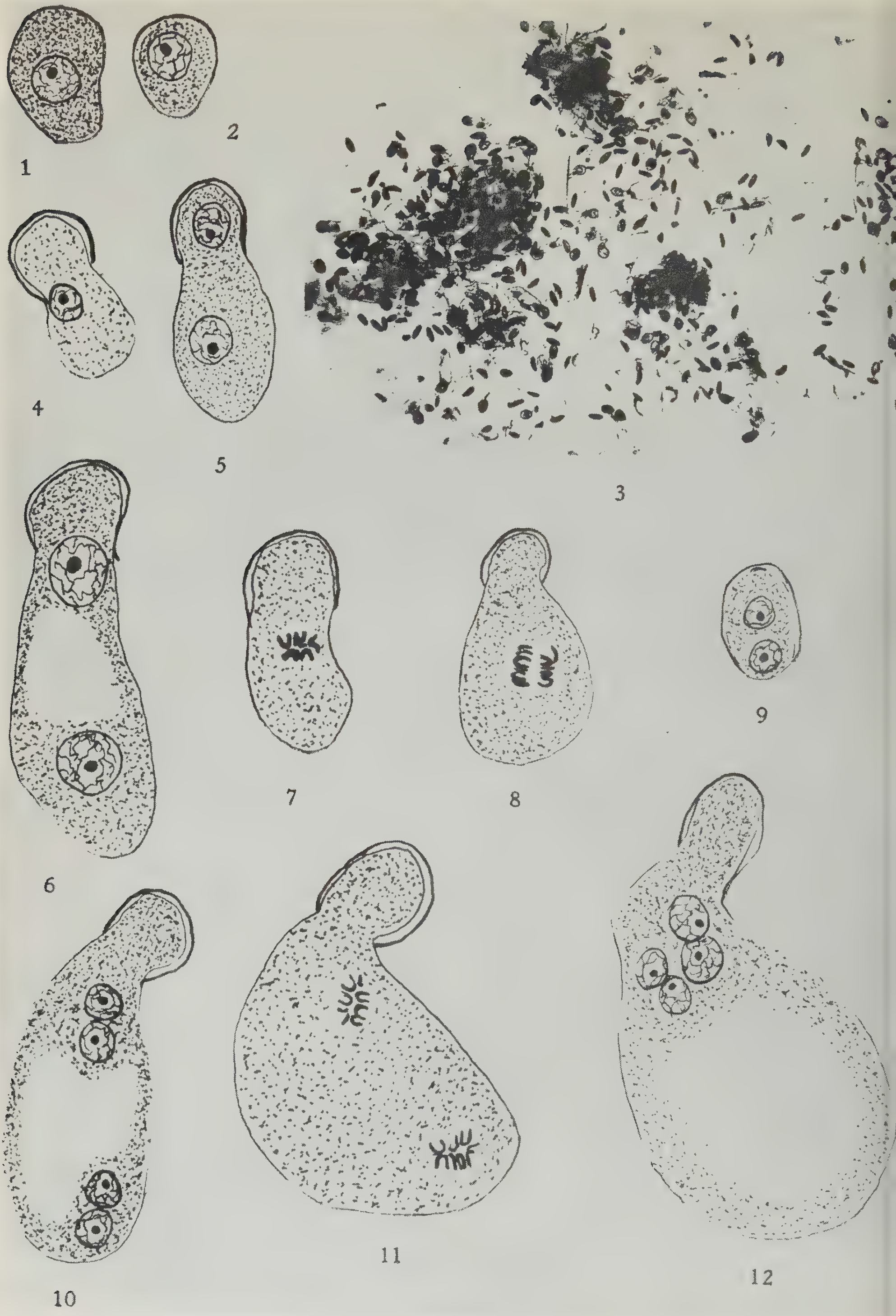
Fig. 1. Abnormal giant pollen grain from treated bulbs.
Fig. 2. Normal pollen grain from untreated bulb.
Fig. 3. Photomicrograph showing about 60 per cent. pollen grains producing embryo-sacs.
Fig. 4. Giant pollen grain, tube formed.
Figs. 5 and 6. Different stages of the two-nucleate pollen embryo-sacs.
Fig. 7. Metaphase of the first division in pollen grain.
Fig. 8. Anaphase of this division.
Fig. 9. Two-nucleate pollen grain, tube not yet formed.
Fig. 10. Four-nucleate pollen embryo-sac.
Fig. 11. Metaphase of the second division.
Fig. 12. Four nuclei near the centre of the tube.
Fig. 13. Photomicrograph of pollen embryo-sac showing the prophase of the third division.
Fig. 14. Eight-nucleate pollen embryo-sac.

Fig. 15. Later than 14. Walls have formed round all the nuclei, which have arranged themselves as in a typical embryo-sac.

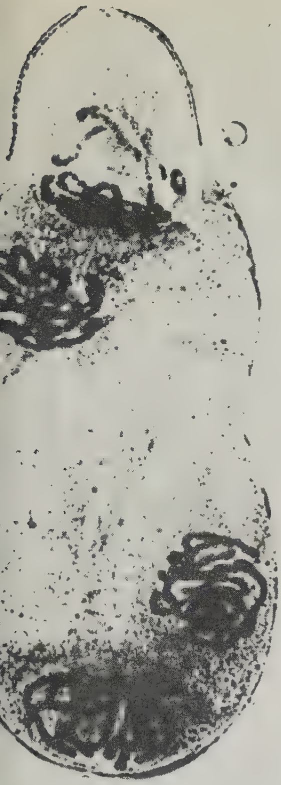
Fig. 16. Irregular arrangement of the eight nuclei.

Fig. 17. Pollen embryo-sac in which the polar nuclei have not migrated and two of the 'micropolar' nuclei have fused.

Fig. 18. Pollen embryo-sac in which two nuclei have fused, two are fusing, and two appear to be about to fuse.



NAITHANI — SEX REVERSAL IN HYACINTHUS.



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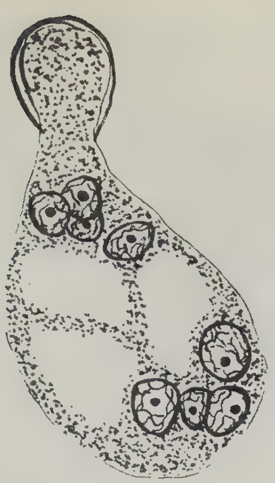

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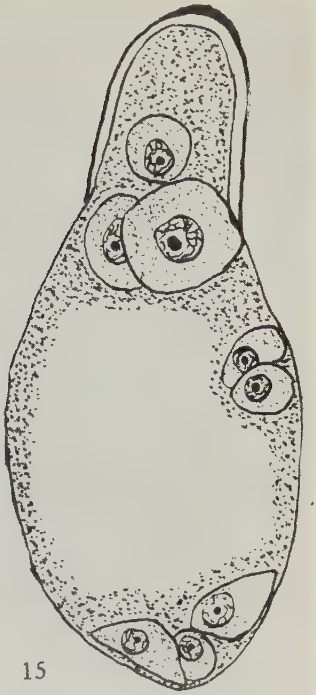

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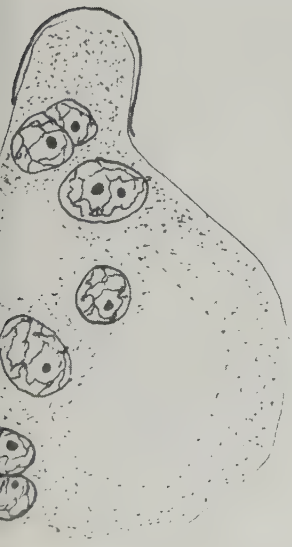

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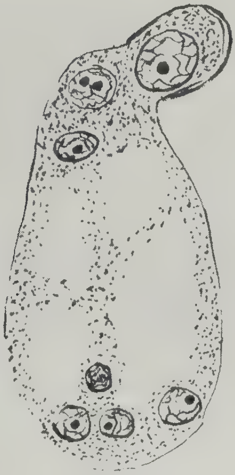

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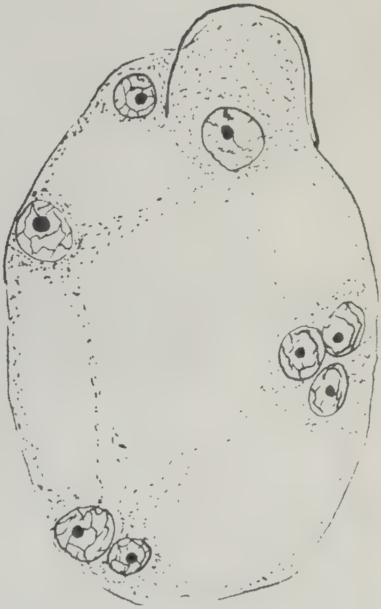

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Studies in the Inheritance of Physiological Characters

IV. Hybrid Vigour in the Tomato

Part 2. Manifestations of Hybrid Vigour during the Flowering Period

BY

L. C. LUCKWILL, B.Sc.

(*From the Department of Botany, The University, Bristol*)

With Plate XVIII and five Figures in the Text

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I. INTRODUCTION

EVER since the rediscovery of Mendel's classical work on plant hybridization the attention of geneticists has been devoted principally to the mode of inheritance of morphological characters such as those chosen for study by Mendel himself. Such morphological characters are the external expression of the physiological processes taking place within the organism, and of recent years an increasing amount of attention has been devoted to the question of the mechanism by which particular genes can produce specific effects of size, shape, or colour. There is an increasing body of evidence that they do so by controlling rates of development.

Sirks (1931), from an extensive genotypic analysis of the Broad Bean, concludes that the size and shape of the various organs are determined by growth-rate factors, and Goldschmidt (1927) has shown how even such phenomena as sex and the colour patterns on lepidopteran wings can be interpreted in terms of rates of development. In the amphipod *Gammarus chevreuxi* it has been shown by Huxley and Ford (1927) that the genes affecting eye colour operate by altering the rate at which melanin is deposited, and Ford (1928) has demonstrated that genetic dwarfing in the same organism is dependent upon a single recessive factor which reduces the growth rate. More recently, Sinnott (1935) has discovered a gene which controls fruit shape in Cucurbita independently of fruit size, and he considers that it does so by controlling the relative rates of growth in two dimensions. These examples are but a selection

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of some of the recent investigations on this aspect of genetics. In fact it is now becoming evident that factors for size, shape, and colour should be interpreted rather as factors influencing rates of developmental processes, the end-points of which are manifest as morphological characters.

In view of these facts, the interest and importance attaching to a direct study of the mode of inheritance of such characters as rates of growth, respiration and carbon assimilation, at once becomes apparent. Such a study was undertaken by Ashby, who in 1930 published the first of a series of papers on the inheritance of physiological characters. In this paper evidence was adduced that the efficiency index in crosses from two strains of maize was inherited in the manner of a simple dominant; that is to say, the efficiency index of the F_1 hybrid was not intermediate between the efficiency indices of the parental forms but was the same as that of one parent. In a subsequent paper (Ashby, 1932) it was shown that assimilation rates and respiration rates were inherited in a similar manner, and in addition evidence was produced of a segregation of efficiency indices in an F_2 population. These investigations have since been repeated (Ashby, 1937) with three different tomato hybrids, two of which exhibited marked heterosis, and in each instance the results were found to confirm those presented in the earlier papers.

Closely linked with the question of the inheritance of growth rates is the problem of hybrid vigour. According to the current hypothesis to account for this phenomenon proposed by Jones (1917), we should expect to find in an F_1 population showing heterosis a higher relative growth rate than in either of the parental forms. This, however, is not in accordance with the data of Ashby,who showed that the hybrid vigour as measured by weight in his material was due, not to any increase in relative growth rate during the period of sampling, but to the fact that the weight of primordia in the hybrid embryos was greater. In other words, the increased weight of the hybrid was nothing more than the maintenance of an initial advantage in size (weight) of primordia. There is at present no authentic instance in which a higher relative rate of growth in an F_1 hybrid has been demonstrated, although as Ashby has predicted (1932), it is probable that such instances will be discovered. Malinowski (1935) in a recent publication on hybrid vigour in Phaseolus states that the rate of growth of the terminal leaflets of the F_1 plants was found to exceed that of the parental varieties. He deals, however, only with arithmetic or absolute growth rates, and his data are too meagre to be calculated on a logarithmic basis. East (1936), in a recent review of the problem of heterosis, in which he calls in question the work of Ashby, maintains that the increased vigour so frequently observable in first generation hybrids is due to an increased relative growth rate of the hybrid plants over that of their parents, but as he produces no data to support it this assertion need not be considered further. Sprague (1936) has repeated Ashby's work on maize, and he also finds no greater relative growth rate in the hybrid from the second week after germination.

Lindstrom (1935) published the results of an experiment which he considered to be 'a test of the Ashby hypothesis of heterosis'. The experiment need not be considered here, for as Ashby has pointed out (1936), it has no bearing on the problem in question. In his paper, however, Lindstrom makes the following statement: 'It may well be that the vigorous F_1 hybrid makes its greater relative growth . . . at the later stages of its history.' Whether this is so or not cannot be decided from the data already published, because Ashby confined his observations to that part of the life-cycle between germination and the onset of flowering. The purpose of the present paper is to discuss the manifestations of heterosis during the flowering period. It must be emphasized, however, that, even if Lindstrom's supposition were correct, a higher relative rate of growth during senescence could not explain the hybrid vigour which is present in the period before flowering, although Lindstrom seems to imply that it could.

In this paper is given an account of an experiment carried out in 1936 with tomatoes, in which a population of F_1 hybrid plants was grown side by side with populations of the two parents. The hybrid was inter-specific, and exhibited marked heterosis. The objects of the experiment were: (1) To study the inheritance of growth rates in the F_1 generation during the flowering period, and thus discover whether Lindstrom's supposition was correct. (2) To investigate the manifestations of heterosis during this period of the life-cycle. (3) To study the inheritance of various other characters such as internode length and time of flowering.

II. Description of the Experiment

1. *Material.*

The strains used in the experiment were:

A = *Lycopersicum racemigerum*, a tall South American species apparently homozygous for all the dominant genes described by MacArthur (1934) for the tomato, or possibly for homologues of these.

B = *Lycopersicum esculentum* var. 'Chinaman', a peculiar dwarf variety of the tomato which is cultivated by the Chinese for the Australian market. It is homozygous for the following recessives: *br* = brachytic stem having short internodes; *wt* = wilty foliage, a condition in which the margins of the leaves are rolled inwards; *f* = fasciated fruit shape as opposed to regular; *n* = nipple tipped fruits.

C = *L. racemigerum* × 'Chinaman', the F_1 hybrid between the above two types.

2. *Method.*

The seeds were sown singly in 3-inch pots on January 29, 1936, and were kept in a propagating house. Germination started about two weeks later and was spread over a further period of two weeks. This long period of germination and the consequent heterogeneity of the populations is probably responsible for the somewhat high sampling errors (see p. 405). The order in which germination occurred was not observed in this experiment, but a subsidiary experiment was later done to determine this. About twenty seeds of each of the three strains were sown at a uniform depth in a seed-box, which was then placed in a propagating frame at a temperature of about 70° F. Table I gives the percentage of seedlings of each strain showing above ground on each successive day. It can be seen from the table that the order of germination is as follows: 1. Hybrid, 2. *L. racemigerum*, 3. 'Chinaman'. This subsidiary experiment, although it was carried out under different conditions, and with seeds of the reciprocal hybrid ('Chinaman' × *L. rac.*), gives an indication of the order of germination of the three strains in the main experiment. Such earlier germination of hybrid seeds, however, is not general; unpublished data show that in certain other tomato crosses showing heterosis, the hybrid germinates later than either of its parents.

TABLE I

Germination Rates. Number of Seedlings Showing above Ground on Successive Days as Percentage of Total Number

Days from sowing .	4.	5.	6.	7.
L. racemigerum . . .	—	50	90	100
'Chinaman'	—	20	75	100
F_1 'Chinaman' × *L. racemigerum* . . .	25	100	100	100

The population of seedlings was sampled twice, at five and eight weeks after sowing. At each of these samplings five plants were taken at random from each of the three strains, and their leaf area, leaf number, and total dry weight determined. Immediately after the second sampling the young plants were planted out 18 in. apart in a bed in the greenhouse. The method employed was to knock the plants out of their pots and plant them complete with the ball of soil, so as to disturb the roots as little as possible. From that time onwards the plants were grown under normal cultural conditions, except that the side branches were not removed as is done in commercial cultivation. In order that any environmental differences due to the position in the greenhouse could be estimated by statistical treatment of the results, the experiment was arranged in three blocks. In each block there were twelve rows, and in each row three plants of the same strain. The arrangement of the rows within the blocks was entirely at random, and was determined by drawing tickets marked *A*, *B*, or *C* out of a hat. Altogether then, thirty-six plants of each strain were grown, allowing for six samples of six plants each. Of these six plants, two came from each block, and the variance between these two individuals was taken as a measure of the experimental error. The particular plants to be removed at each sampling was prearranged so as to ensure that the population should be thinned out evenly, and thus prevent secondary effects due to overcrowding and shading.

Flowering began approximately twelve weeks after sowing, and the populations were sampled after 13, 15, 17, 19, and 23 weeks. It was originally intended to take another sample after twenty-seven weeks, but by that time the plants had grown so large that it was considered impracticable to do so, as facilities for drying material were somewhat limited. In sampling, only the overground portions of the plants were taken into account, as other workers have found a high correlation between shoot weight and root weight in the tomato (Fabergé, 1936). The plants to be sampled were cut off at ground-level and brought into the laboratory where various morphological observations and measurements were made. Leaf area was at first determined by the use of a photo-electric phyllometer (Ashby, 1937), but for the last three samples this was found to be impracticable and the following method of estimating leaf area was devised. A certain number of leaves was measured on the phyllometer and a graph was constructed showing the relationship between leaf area and fresh weight of leaves. In order to estimate the leaf area of any plant it was then only necessary to find the fresh weight of its leaves and to read off the corresponding value for area on the graph. The maximum errors involved in this procedure are of the order of 20 per cent., and the mean error is about 10 per cent. (see Table II); the differences in leaf area are of the order of 60–100 per cent. so that the method is quite satisfactory for this type of experiment.

TABLE II

Areas of Tomato Leaves in sq. cm., as Measured by a Phyllometer and as Calculated on a Fresh Weight Basis

Measured.	Calculated.	Per cent. Error.
675	780	+15·5
980	1200	+22·5
1175	1170	−0·4
1230	1175	−4·4
1780	1950	+9·5
3970	3800	−4·3

Mean per cent. error = 9·43.

The plants were then dried for about twenty hours in steam ovens at a temperature of 90°–100° C., and the dry weights of both stems and leaves were found.

The results of the experiment were treated by the method of analysis of variance. Summaries only of the statistical tables are given in the main text, but a full account of the statistical methods, together with tables, will be found in the Appendix on pp. 404–7.

3. *Data.*

Table III contains a summary of the principal experimental data. Each figure in the table is the mean of six values, except in the case of the two seedling samples (fifth and eighth week), where only five individuals of each strain were measured. These data are only given for the sake of comparison with data from the latter part of the life-cycle.

The experiment was continued up to the twenty-third week after sowing. By this time the tall parent had reached a height of over 2 metres, but the dwarf 'Chinaman' measured less than 1 metre in height and had practically stopped growing. The photographs reproduced in Pl. XVIII, taken twenty-six weeks after sowing, show the comparative height of the plants. The remaining eighteen plants were left until the middle of September which was about thirty weeks from the date of sowing, when they were again examined. Most of the dwarfs were found to be dead, but the tall *L. racemigerum* and the hybrid were still growing vigorously and producing new inflorescences, and apparently would have gone on doing so for a considerable time had they been kept under suitable conditions. A typical hybrid plant which was cut down had a fresh weight of 16,800 gm. and measured 4 metres in height. There is no evidence from this experiment that the vigorous F_1 hybrid exceeds its tall parent in the duration of the period over which growth is continued.

TABLE III

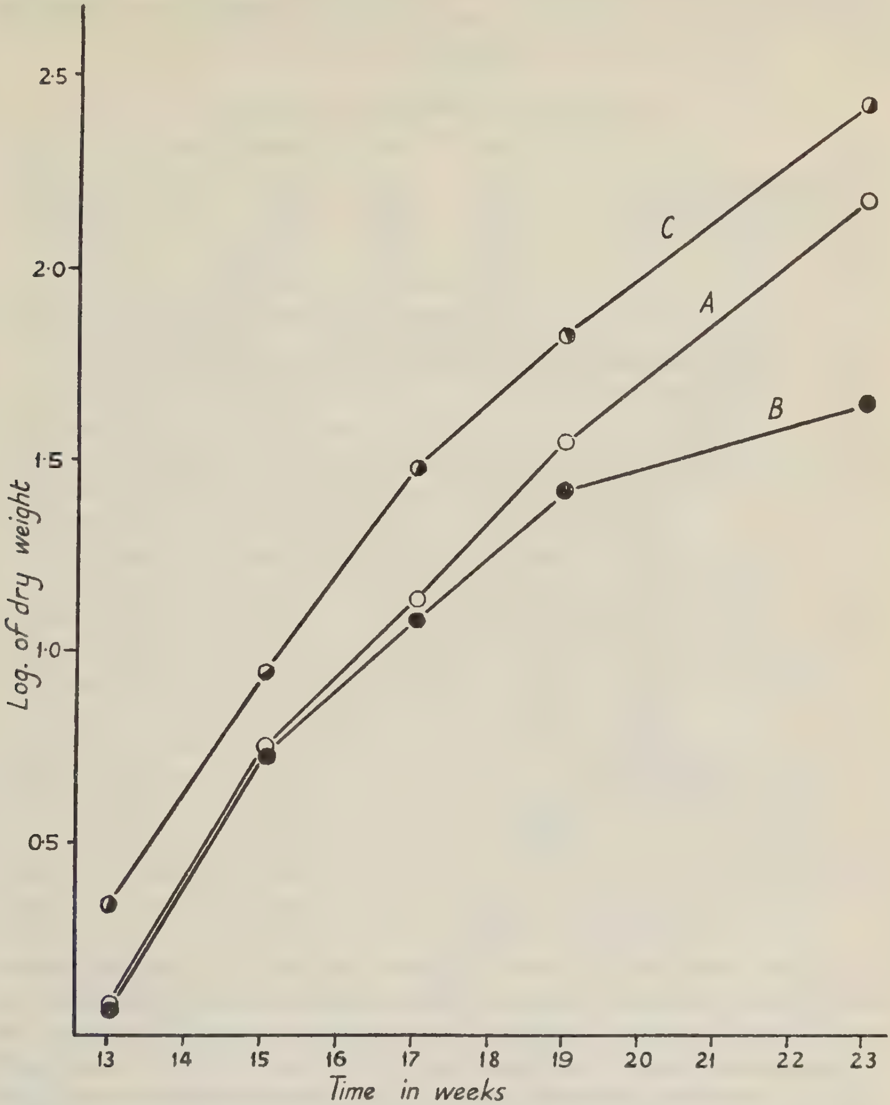
Principal Experimental Data

Age of sample (weeks).		5.	8.	13.	15.	17.	19.	23.
Total dry wt. (gm.).	A.	0·0085	0·0942	1·17	5·60	13·85	36·16	157·1
	B.	0·0086	0·1307	1·14	5·38	12·33	27·05	47·1
	C.	0·0134	0·1638	2·16	8·81	30·22	69·43	274·3
Dry wt. stems (gm.).	A.	—	—	0·34	1·69	5·82	17·4	90·5
	B.	—	—	0·28	1·39	3·58	8·3	14·9
	C.	—	—	0·47	2·04	9·72	27·3	129·6
Dry wt. leaves (gm.).	A.	—	—	0·83	3·91	7·94	18·7	66·6
	B.	—	—	0·86	3·99	8·75	18·7	32·1
	C.	—	—	1·69	6·77	20·49	42·2	144·6
Leaf number.	A.	2·0	5·6	11·0	41·8	80·3	132·7	284·1
	B.	1·2	5·6	9·5	33·8	62·3	91·3	99·1
	C.	2·2	5·8	12·0	45·8	91·5	159·8	319·3
Total leaf area (sq. cm.).	A.	1·92	27·8	173	723	1512	3737	13906
	B.	0·94	32·0	158	752	1603	4547	8044
	C.	3·06	34·8	311	1236	3502	9083	33283
No. of inflorescences.	A.	—	—	—[1]	4·2	12·8	26·0	69·0
	B.	—	—	—	2·3	6·8	12·1	19·0
	C.	—	—	—	3·3	15·6	24·6	77·4
No. of inflorescences with open flowers.	A.	—	—	0·83	2·16	4·33	8·5	42·0
	B.	—	—	0	1·0	1·50	3·2	9·5
	C.	—	—	0	1·5	3·66	9·66	52·2
Height (cm.).	A.	—	—	19·3	—	—	97	220
	B.	—	—	13·0	—	—	64	73
	C.	—	—	17·7	—	—	113	201

[1] Observations of total number of inflorescences were not made on this occasion.

The results of the experiment will now be presented in more detail:

Total dry weight and efficiency index. It has been shown in a previous paper of this series (Ashby, 1937) that the efficiency index in the tomato remains practically constant from germination to the onset of flowering. During the flowering period it was found that the efficiency indices of all three strains fell continuously (see Table IV). The curves showing the logarithmic increase of dry weight with time are given in Text-fig. 1, and it is obvious from inspection of these curves that, over the duration of the experiment, the relative growth rates of the tall parent and the hybrid are very similar and differ from that of the dwarf parent. Moreover, calculations show that the



TEXT-FIG. 1. Logarithmic growth curves of the three strains used in the experiment during the flowering period. A = tall parent; B = dwarf parent; C = F_1 hybrid.

mean efficiency indices of *L. racemigerum* and the hybrid, from the thirteenth to the twenty-third week, are the same to the third decimal place (Table IV). Such close agreement, however, is probably not as significant as would at first appear, since when the mean efficiency indices for the periods between successive samplings are calculated, the values show considerable deviations.

TABLE IV

Mean Efficiency Indices During Senescence

Weeks	13–15.	15–17.	17–19.	19–23.	Mean.
A. L. racemigerum	0·340	0·196	0·208	0·159	0·226
B. L. escul. var. 'Chinaman'	0·290	0·142	0·109	0·082	0·156
C. F_1 *L. rac.* × 'Chinaman'	0·305	0·267	0·181	0·149	0·226

TABLE V

Analysis of Variance of Logarithms of Total Dry Weights

'S' means that the variance is significant on the 1 per cent. point of *z*.

Variance due to:	
Time	S
Block	—
Strain	S
Between *A* and *B*	S
13th to 15th week	—
15th ,, 17th ,,	—
17th ,, 19th ,,	—
19th ,, 23rd ,,	S
Between *B* and *C*	S
,, *A* and *C*	S
13th to 15th week	S
15th ,, 17th ,,	S
17th ,, 19th ,,	S
19th ,, 23rd ,,	S
Interactions:	
Time × strain	S
Between *A* and *B*	S
,, *B* and *C*	S
,, *A* and *C*	—
Strain × block	—
Block × time	—
Strain × block × time	—

In Table V is given a summary of the results of a statistical analysis of the data for total dry weight. The full analysis will be found in the Appendix (p. 405).

In this experiment the variances due to block, and the interactions of block with strain and time are not significant. In other words, there are no differential environmental effects which need be taken into account. The two variances in which we are chiefly interested are those due to strain and the interaction of strain with time, both of which are significant. The variance due to time is, of course, highly significant, since the plants were growing.

In Text-fig. 1 the growth curve of the hybrid (*C*) lies above that of *L. racemigerum* (*A*), thus showing that at any given time the total dry weight of the hybrid was greater than that of the tall parent, i.e. there was marked weight heterosis in the F_1 generation. That this difference is a significant one can be seen from the partition of strain variance given in Table V. The same analysis indicates, as would be expected, a significant difference between the total dry weights of the tall and the dwarf parents (i.e. between *A* and *B*), but further partition shows that this difference does not arise until after the nineteenth week. The strain variance between *A* and *C*, on the other hand, is significant throughout the duration of the experiment.

TABLE VI

Mean Relative Growth Rates of Stems and Leaves

Weeks.	13–15.	15–17.	17–19.	19–23.	Mean.
(a) *Leaves*:					
A. L. racemigerum . .	0·336	0·154	0·186	0·138	0·203
B. L. escul. var. 'Chinaman'	0·333	0·170	0·165	0·058	0·181
C. F_1 *L. rac.*×'Chinaman' .	0·301	0·240	0·156	0·134	0·208
(b) *Stems*:					
A. L. racemigerum . .	0·348	0·268	0·237	0·179	0·258
B. L. escul. var. 'Chinaman'	0·348	0·205	0·182	0·063	0·199
C. F_1 *L. rac.*×'Chinaman' .	0·319	0·339	0·224	0·169	0·263

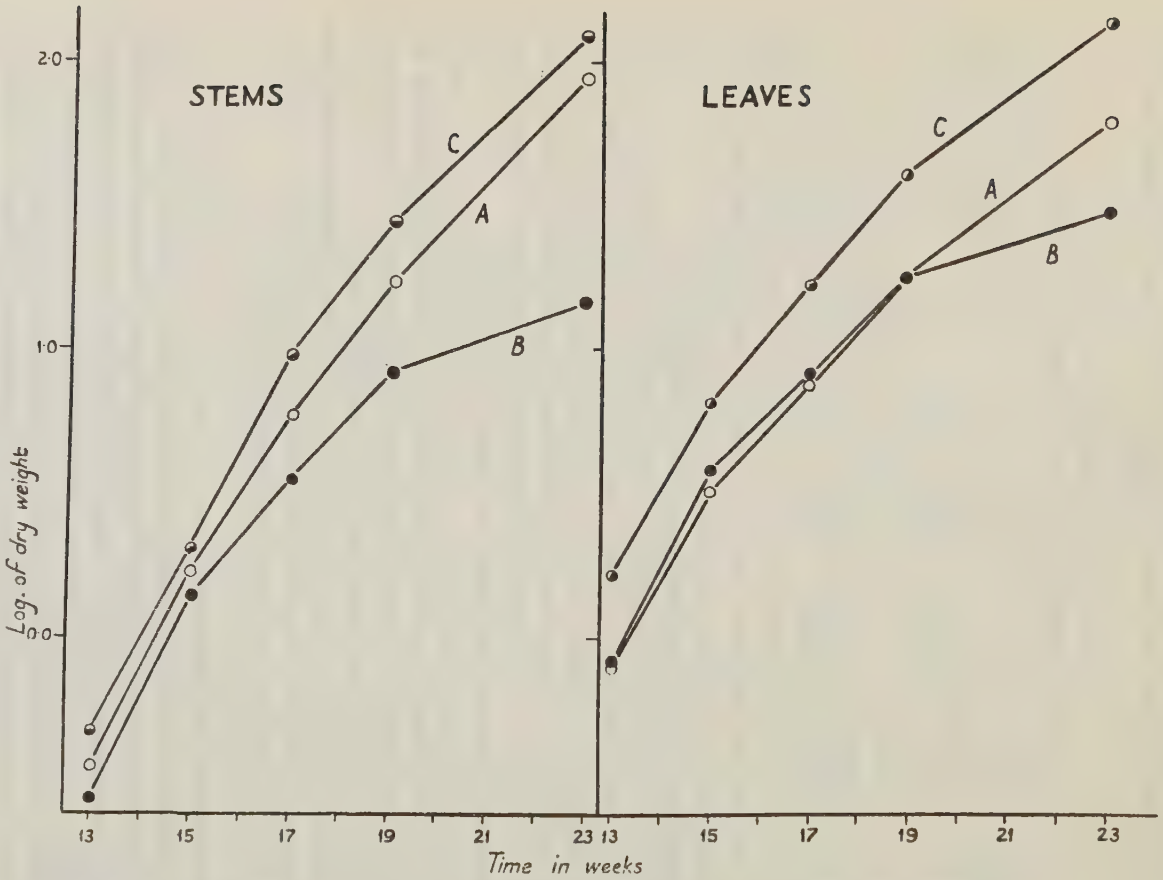
The most interesting feature of the analysis is the significance of the strain × time interaction. It indicates differences in relative growth rates. Partition of this variance, however, shows that such differences are entirely accounted for by the variance between the two parents (*A* and *B*), and between the dwarf parent and the hybrid (*B* and *C*). *There is no significant time × strain interaction between the tall parent and the hybrid* (*A and C*).

We may say therefore, from the results of this analysis, that from the beginning of the flowering period up to the twenty-third week after sowing, the hybrid does not differ from its taller parent either in its relative rate of growth or in the length of the period over which growth is maintained, although its absolute dry weight is over 40 per cent. higher. Hence this experiment does not support the view expressed by Lindstrom (1935) that the 'hybrid makes its greater relative growth at the later stages of its history'.

Dry weight of stems and leaves.

The mean relative growth rates of stems and leaves as calculated on dry weight increase are given in Table VI. In all three strains the stems increased in dry weight more rapidly than the leaves, but in each instance there is a close similarity between the mean relative growth rates of *L. racemigerum* and the hybrid.

The curves showing the logarithmic increase in dry weight of stems and leaves (Text-fig. 2) are very similar in general form to those for total dry weight described above, and for this reason they were not analysed in such detail. It can be seen from the curves that heterosis is present both in the stems and in the leaves, as in each instance the hybrid curve lies above that for the tall parent. The distance between the curves, however, is over twice



TEXT-FIG. 2. Curves showing the dry weight increase of stems and leaves during the flowering period. A = tall parent; B = dwarf parent; C = F_1 hybrid.

as great in the case of the leaves as in the case of the stems, so that heterosis is not manifested equally in both these organs. Actually 68·8 per cent. of the increased weight of the hybrid over the tall parent is due to increased leaf weight, and only 31·2 per cent. to stem. It can also be seen from Text-fig. 2 that the slopes of the curves for the hybrid do not differ appreciably from those of the curves for the tall parent, so that heterosis is not accompanied by any increase in relative growth rate.

TABLE VII

Mean Relative Growth Rates Calculated as Height

Weeks:	13–19.	19–23.
A. L. racemigerum	0·116	0·088
B. L. escul. var. 'Chinaman'	0·115	0·015
C. F_1 *L. rac.*×'Chinaman'	0·117	0·062

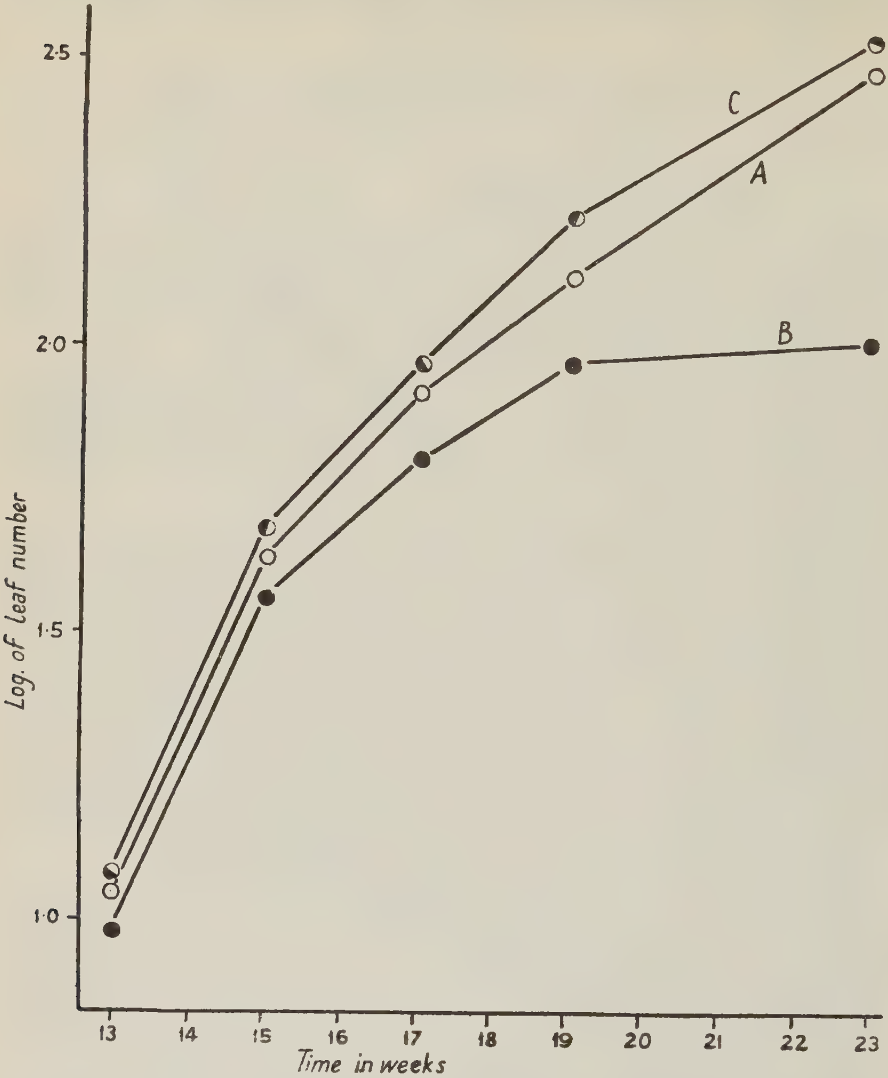
It has already been pointed out that the growth curves for the two parents diverge from the fifteenth week onwards, but inspection of Text-fig. 2 reveals that this divergence is at first due entirely to a fall in the growth rate of the stem of the dwarf parent, the curves for the leaves being practically concurrent up to the nineteenth week.

Differential growth ratio. The relation between the sizes of two growing organs of an animal or plant is given by the equation $y = bx^k$ (Huxley, 1932), where x and y are the respective sizes of the two organs at any given time, and k is the differential growth ratio between them. k is frequently found to be a constant and is given by the slope of the curve when log x is plotted against log y. The fractional coefficient, b, is a constant of no particular biological significance. This formula has been fairly widely employed in the study of animal growth, but its application to plants has so far been limited to the work of Pearsall (1927), who calculated k between stem and root weight of a number of plants and also between petiole length and lamina diameter in *Tropaeolum*. He found in every case that k remained constant.

In the present instance log-stem weight was plotted against log-leaf weight, and straight lines were obtained (Text-fig. 4). This means, that over the duration of the experiment, k was a constant for each of the three strains. It can also be seen from Text-fig. 4 that the value of k for 'Chinaman' is somewhat lower than that for *L. racemigerum*, whilst k for the hybrid was almost exactly the same as that for *L. racemigerum*. The actual values of k, measured graphically, are as follows: *L. rac.* = 1·32; 'Chinaman' = 1·06; F_1 hybrid = 1·30. Unfortunately it was not possible to test the significance of these results, although it seems clear that this physiological character too, is inherited in F_1 in the manner of a dominant, being of the same magnitude in a hybrid showing heterosis as in one of the parents.

Height. Measurements of height were made at the thirteenth, nineteenth, and twenty-third week samplings, and the mean relative growth rates calculated as height are given in Table VII.

From the thirteenth to the nineteenth week the relative growth rates of all three strains as measured by height are the same, although one parent was tall and the other dwarf. After the nineteenth week a rapid falling off in relative growth rate occurs, but it will be observed that in the dwarf parent this diminishes much more rapidly than in the tall parent or the hybrid. The dwarf 'Chinaman' has, in fact, almost stopped growing in height by the nineteenth week.



TEXT-FIG. 3. Logarithms of leaf number plotted against time, during the flowering period. *A* = tall parent; *B* = dwarf parent; *C* = F_1 hybrid.

There are also absolute differences in height between the strains (Table III). The dwarf parent is shorter than the tall parent from the thirteenth week onwards, but there is no significant difference between the tall parent and the hybrid in this respect throughout the duration of the experiment, i.e. in this instance there is no height-heterosis.

Leaf number. In the experiments of Ashby (1937) on the pre-flowering period of the life-cycle in the tomato, leaf number was found to increase in a linear manner with time. It was also found that heterosis was accompanied by an increase in leaf number of the hybrid over its parents, which increase was shown to be due 'entirely to the fact that the first leaf of the hybrid unfolds slightly earlier than the first leaves of either of its parents'.

TABLE VIII

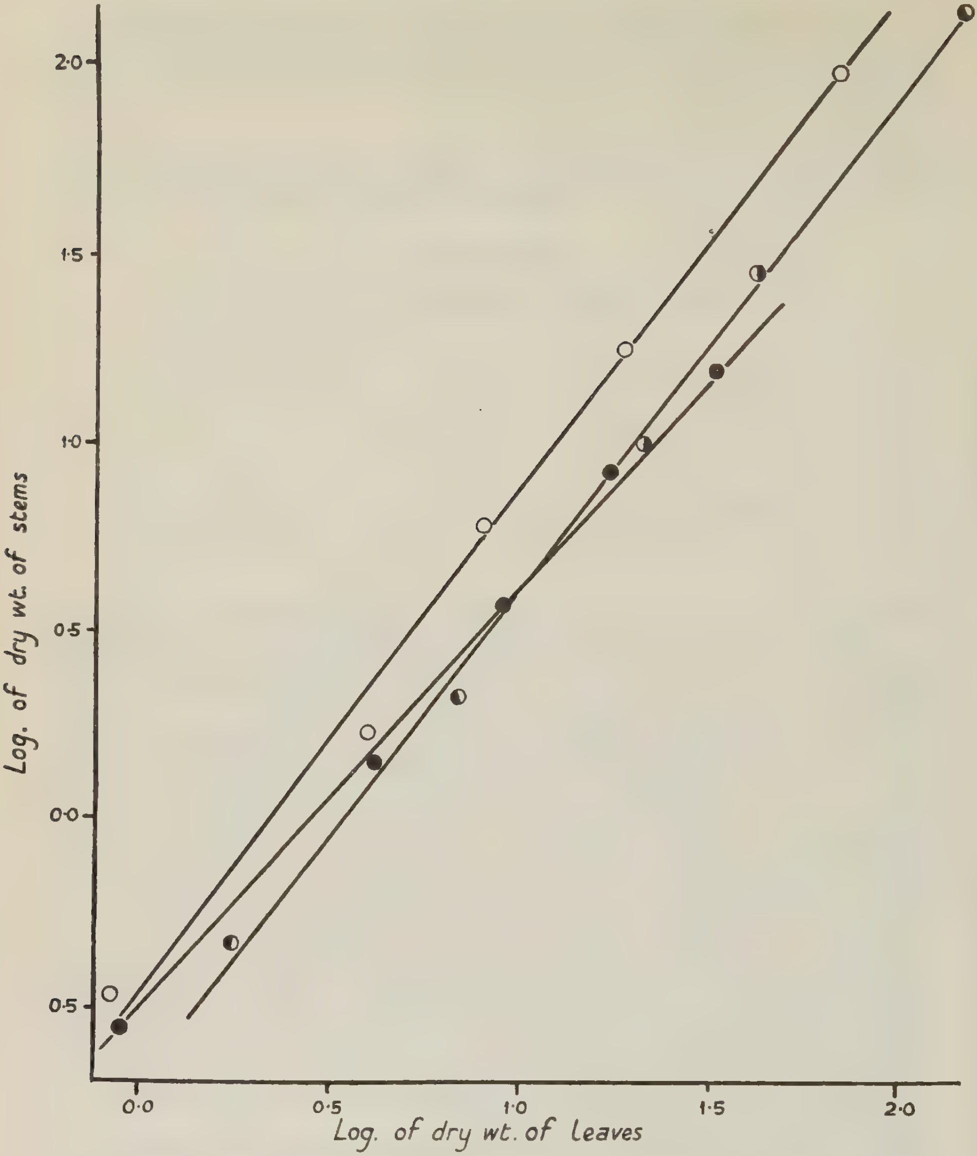
Analysis of Variance of Logarithms of Leaf Number

	1 per cent.	5 per cent.
Variance due to:	points of 'z'.	
Strain	S	S
Between A and B	S	S
,, B ,, C	S	S
,, A ,, C	—	S
Strain × time	S	S
Between A and B	S	S
,, B ,, C	S	S
,, A ,, C	—	—

During the flowering period the relationship of leaf number to time was found to be entirely different from that in the preflowering period. The curves are linear up to the thirteenth week, at which time they suddenly ascend and become approximately exponential. In Text-fig. 3 leaf number is plotted logarithmically against time from the thirteenth to the twenty-third week, and it will be observed that the curves are similar in general form to those for dry weight. Here again, r is not a constant, but falls continuously during the flowering period. The explanation of this approximate exponential increase in leaf number is in the branching of the plants. The rate of production of leaves is of course dependent upon the rate of production of laterals. Lateral branches are first produced at about the same time as the first inflorescence, and since the rate of production of laterals depends on the number already present, the increase will be exponential.

The data for leaf number during the flowering period were treated by the method of analysis of variance, and a summary of the results of the analysis are given in Table VIII. The full analysis will be found on pp. 405–6 of the Appendix.

It is evident from Text-fig. 3 that the curve for the hybrid lies slightly above that for *L. racemigerum*, although the analysis shows that this difference is not significant on the 1 per cent. point of 'z'. It is significant, however, on the 5 per cent. point, so that there is quite a high probability (at least 20 to 1)



TEXT-FIG. 4. Dry weight of stems plotted against dry weight of leaves, both logarithmically. White circles = tall parent; black circles = dwarf parent; half-white circles = F_1 hybrid. The slopes of the curves are the differential growth ratios of the three strains.

that the leaf number of the hybrid is significantly greater than the leaf numbers of either of its parents during this part of the life-cycle. This higher leaf number, however, is not brought about through a higher relative rate of production of leaves, because we see from Table VIII that there is no significant variance due to the interaction of strain and time between the tall parent (*A*) and the hybrid (*C*). It must be due to the maintenance of some 'initial' advantage already present at the thirteenth week. It seems probable, although we have no data on this point, that lateral branching begins in the hybrid at a slightly earlier date than in either of the parent varieties. This would account for the observed 'initial' advantage in leaf number, since, as pointed out above, leaf number depends on the stage of development of the laterals.

Number of inflorescences. After the appearance of the first inflorescence, all three strains produce inflorescences regularly at every third node, so that the rates of production of inflorescences and of leaves are inter-dependent. It has been shown above that the hybrid produces leaves at the same relative rate as *L. racemigerum*, and it follows from this that it also produces inflorescences at the same relative rate. Owing, however, to the fact that the first inflorescence of the hybrid is produced at the ninth node, and consequently appears somewhat later than the first inflorescence of *L. racemigerum* which is produced at the sixth node, the number of inflorescences on the hybrid is not greater than the number on the tall parent, at least during the first ten weeks of the flowering period (see Table III). Even after twenty-three weeks from the date of sowing, although the hybrid appears from the figures in the table to have some slight advantage in inflorescence number, the differences are not significant. The total number of inflorescences on the three strains at the twenty-third week sampling were as follows:

L. racemigerum = 69·0±7·66; 'Chinaman' = 19·0±3·09; F_1 hybrid = 77·4±11·15.

The view has already been expressed that the heterogeneity of the populations, which is responsible for these very high standard errors resulted from the extended period over which germination occurred (p. 381).

Leaf size. It has already been mentioned that 68·8 per cent. of the increased weight of the hybrid over its taller parent is due to increased leaf weight, and since the number of leaves in the hybrid is only about 10 per cent. greater than in *L. racemigerum*, it follows that the size of the individual leaves of the hybrid must be greater. That this is actually the case can be seen from Table IX, in which is given the mean weight and the mean area per leaf of all the leaves measured, on the three strains from the thirteenth to the twenty-third week.

TABLE IX

Mean Weight and Mean Area per Leaf

	A.	*B.*	*C.*	
Mean dry weight .	0·129	0·183	0·246	gm.
Mean area . .	19·2	28·8	37·1	sq. cm.

At the thirteenth week measurements were also made of the length of the fourth and fifth leaves on every plant, and the following mean values were obtained:

4th leaf A = 10·3±0·92 cm.
B = 12·0±0·51 „
C = 15·0±1·06 „
5th leaf A = 11·0±0·68 „
B = 13·0±0·58 „
C = 17·1±1·45 „

In both instances the leaf sizes of the two parents differ significantly, the dwarf 'Chinaman' (B) having larger leaves than the tall *L. racemigerum* (A). The leaves of the hybrid are significantly larger than those of either parent, their mean area being approximately twice as great as the mean area of the leaves of *L. racemigerum*. The large size of the leaves is, in fact, one of the most striking manifestations of heterosis in this particular hybrid, as can be seen from the photographs reproduced in Pl. XVIII.

In order to determine whether these size differences were due to differences in the number or in the size of the cells, measurements were made of cell size in the epidermis of leaves of plants of each of the three strains. Leaves of approximately equal age were used and the areas of 100 cells of the upper epidermis of each were found by a projection method. The results of this investigation are recorded in Table X.

TABLE X

Cell Size and Leaf Size

	L. rac.	'Chinaman'.	F_1 hybrid.
Mean leaf area (sq. cm.) . . .	19·2	28·8	37·1
Mean area of epidermal cells (arbitrary units)	34·5±0·84	64·6±3·92	30·1±1·34
Ratio leaf size/cell size	0·55	0·44	1·23

It can be seen from the table that the cells in the leaves of 'Chinaman' are almost twice as large as those in *L. racemigerum*. Those of the hybrid leaves, however, are slightly smaller than in *L. racemigerum*, and this difference, although small, is a significant one. It is probably due to the fact that the leaves on which the measurements were made were not at precisely the same stage of development. The ratio of leaf size to cell size, also recorded in the table, gives a measure of the relative number of cells per leaf in each of the three strains. The ratio is approximately the same in *L. racemigerum* and 'Chinaman', so that the larger leaves of the latter are entirely accounted for by the larger size of the individual cells. The increased size of the leaves of the hybrid as compared with those of *L. racemigerum*, however, is due, not to any increase in cell size, but to an increased number of cells in each leaf.

There are three possible ways in which this increase in the number of cells could have arisen. These are: (i) a higher rate of cell division in the leaves of the hybrid as compared with the leaves of *L. racemigerum*; (ii) a larger number of cells in the primordia from which the leaves originate; (iii) the duration of the period over which cell division is maintained might be greater in the leaves of the hybrid, or alternatively, the leaves of the hybrid might have been at a later stage of development than the leaves of the tall parent at the time when the measurements were made.

Observations of the time of unfolding of the first leaves were not made in this experiment, although data from a subsidiary experiment (unpublished) indicate that this takes place earlier in the hybrid than in either of the parent strains. This means that the hybrid leaves at the time of sampling were actually at a later stage of development, and part of their increased size must be due to this fact. The advantage, however, is a small one (one to two days), and is by no means sufficient to account for the whole of the observed increase in leaf size.

No direct data are available concerning the rate of growth of individual leaves. It has already been shown, however, that the relative rate of increase of total leaf weight is the same in the hybrid as in the tall parent. It has also been shown that the relative rates of production of leaves are the same in these two strains, and it therefore follows that the relative growth rates of their leaves are the same also. The only other possibility is that the increasd size of the hybrid leaves is due to the fact that they arise from primordia which contain a correspondingly larger number of cells than those which give rise to the leaves of *L. racemigerum*. Up to the present it has not been found possible to confirm this view by direct anatomical observation, although work is at present in progress to determine this point.

Assimilation rate. The mean assimilation rates of the three strains were estimated by the use of the formula (Briggs, Kidd, and West, 1920):

$$\text{Assimilation rate} = \frac{W_t - W_0}{\bar{A}}$$

where

$$\bar{A} = \frac{A_t - A_0}{\log_e A_t - \log_e A_0}$$

and W_0 = original dry weight; W_t = dry weight after time t; A_0 = original leaf area; A_t = leaf area after time t.

Table XI gives the mean values of the assimilation rates as calculated on this formula over the periods between successive samplings. The values, however, are necessarily only approximations. The total leaf area is by no means a precise measure of the effective amount of assimilating surface, especially in large plants where there is much mutual shading of the leaves.

TABLE XI

Mean Assimilation Rates in gm. per sq. dm. per week

Weeks:	5–8.	8–13.	13–15.	15–17.	17–19.	19–23.	Means.
A	0·294	0·270	0·575	0·385	0·452	0·390	0·394
B	0·463	0·256	0·555	0·309	0·261	0·082	0·321
C	0·383	0·318	0·496	0·492	0·335	0·275	0·383

After an initial drop, due no doubt to the fact that the plants were transplanted at the eighth week, the assimilation rates of all three strains increase up to the fifteenth week after sowing. Up to this time there is little absolute difference between the strains. From the fifteenth week onwards the rates show a steady decline, but that of 'Chinaman' falls off more rapidly than either *L. racemigerum* or the hybrid. When the mean assimilation rates over the whole period of growth are considered, it will be seen from Table XI that there is a close correspondence between those of the tall parent (A) and the hybrid (C), whilst that of the dwarf parent (B) is somewhat lower. These results, therefore, confirm the conclusion of Ashby (1932, 1937) that heterosis is not accompanied by any increase in the rate of assimilation in the vigorous F_1 hybrid.

Time of flowering. L. racemigerum produced its first flowers twelve weeks after sowing, and 'Chinaman' came into flower about a fortnight later. The hybrid was intermediate in its time of flowering, commencing at the thirteenth week after sowing.

In the tomato, the time of flowering seems to be connected with the position of the first inflorescence. In *L. racemigerum*, the first inflorescence is produced at the sixth node, in 'Chinaman' not until the twelfth node, and in the hybrid at the ninth node. The approximate time of formation of these nodes can be estimated from the graph of leaf number plotted against time. In the case of *L. racemigerum* the mean time of unfolding of the sixth leaf is fifty-eight days from sowing. The twelfth leaf of 'Chinaman' unfolds on the ninety-third day, and the ninth leaf of the hybrid on the seventy-ninth day. The data for time of flowering are not complete enough to warrant a close comparison of these figures, since they represent only the approximate dates at which flowering commenced, and not the mean time of flowering of the whole population. Nevertheless, it is significant that the order in which the strains flowered is the same as the order in which the first inflorescence-bearing nodes were formed.

An intermediate inheritance of time of flowering was observed also by Mendel (1865) in crosses between early and late flowering peas, and Keeble and Pellew (1910) state that earliness and lateness of flowering seem to be connected with morphological characters, especially internode length. This is confirmed by recent work of Rasmusson (1935).

Mode of branching. The growth of the stem in the tomato is, as Crane (1915) has shown, sympodial, the leading growth being morphologically lateral. In *L. racemigerum*, what appears to be the main stem produces a number of 'laterals' near the base. These 'laterals' almost equal the 'main' stem in size, and are themselves profusely branched. The habit of the dwarf 'Chinaman' is peculiar. The 'main' stem, instead of being of indefinite growth as is the normal condition in the tomato, is terminated at the twelfth node by a very large and fasciated inflorescence. After this has been formed, growth is continued by a limited number of lateral branches, the inflorescences on which are much smaller than the first-formed inflorescence, and appear to set little fruit. The hybrid closely resembles *L. racemigerum* in its mode of branching, the only difference being that it produces a larger number of lateral branches. The view has already been expressed (p. 391) that this is due to the fact that branching begins in the hybrid slightly earlier than in either of the parent strains.

Internode length. The frequency distributions of internode length as measured on the longest shoots of two plants of each strain at the twenty-third week sampling, are given in Table XII.

TABLE XII

Frequency Distributions of Internode Length

Class.	0–3.	3–6.	6–9.	9–12.	12–15.	15–18.	18–21.	21–24.	24–27. cm.	Means.
A	21	15	13	7	5	0	3	0	1	6·68±0·679
B	33	12	6	—	—	—	—	—	—	2·90±0·315
C	19	13	16	4	8	1	—	—	—	6·54±0·548

The modal values of the three distributions are the same, but 'Chinaman' differs from *L. racemigerum* in its much smaller range of internode length. Presumably the internode lengths of the two parents were the same at the beginning, but the recessive gene for brachytic stem in 'Chinaman' acted by preventing the later elongation of the internodes such as occurs in the tall parent.

Heterosis, in this instance, is not accompanied by any increase in internode length. There is no significant difference between the tall parent and the hybrid, either in the form of the distribution curve or in the mean internode length.

Size of primordia. The importance of the amount of 'biological capital' as represented by the weight of primordial tissue present in the embryo in the determination of heterosis, has frequently been emphasized by Ashby (1930, 1932, 1937). In the present experiment determinations of embryo weight were made on each of the three strains in the following manner.

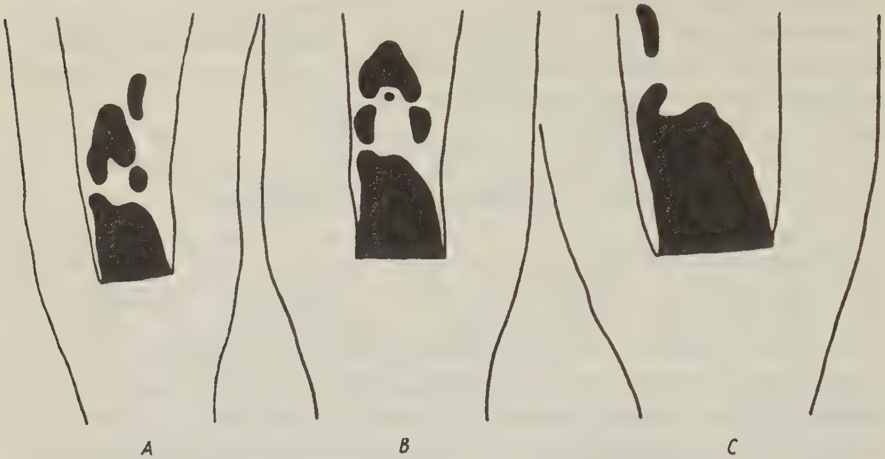
TABLE XIII

Embryo Weights in mg.

L. racemigerum . .	0·656±0·034
L. escul. var. 'Chinaman' .	1·080±0·110
F_1 *L. rac.*×'Chinaman' .	1·387±0·055

About twenty seeds of each strain were soaked in 5 per cent. formalin for two days. The embryos were then dissected out, washed, and dried for three hours in a steam oven at 100° C. The embryos were weighed on a special torsion balance. Up to the time of weighing the embryos were kept in a desiccator, but owing to the rapidity with which weighings could be made, precautions against the absorption of moisture whilst on the balance pan were found to be unnecessary. The values obtained are given in Table XIII.

It is clear from these figures that heterosis is already present in the embryo stage, the F_1 embryos being significantly heavier than those of either parent. The amount of heterosis present in the embryo, as measured by the logarithmic difference between the dry weights of the hybrid and the tall parent is 0·325, whereas the amount present after thirteen weeks was only 0·266, and after twenty-three weeks 0·242. That such discrepancies should exist, however, is not surprising, since in the tomato the resting embryo consists almost entirely of radicle, hypocotyl, and cotyledons. The primordium which gives rise to the shoot is represented only by a small group of cells, which at this early stage it is impossible to dissect out and weigh separately.



TEXT-FIG. 5. Median longitudinal sections through young shoot primordia of the same age. A = tall parent; B = dwarf parent; C = F_1 hybrid. The primordia tissue is blackened.

Although it was not found practicable to measure the shoot primordia in resting embryos, direct evidence was obtained of the presence of heterosis in primordia which were somewhat older. A few seeds of each of the three strains were sown in a propagating frame, and when the seedlings had reached the stage at which their cotyledons were expanded but no foliage leaves were yet showing, they were fixed in weak Flemming's solution. Vertical sections in the plane of the cotyledons were then made and the median sections through the plumules were selected and drawn. The drawings are reproduced in Text-fig. 5, from which it is at once apparent that heterosis is present, even at this early stage. The areas of primordia (non-vacuolated cells) in these sections are as follows: *L. rac.* (A) = 1·25; 'Chinaman' (B) = 1·75; F_1 *L. rac.* × 'Chinaman' (C) = 2·34. The amount of heterosis (log. C — log. A) is 0·272, which is approximately the amount which was observed during the flowering period. Here again we are not justified in making close comparisons, because so far measurements of primordia have been made only on a few plants and not on whole populations. The investigation, however, although only preliminary, does indicate one important fact, viz. the presence of heterosis in very young primordia.

It will be observed from these figures that both the embryo weight and the size of the shoot primordium in 'Chinaman' are greater than the corresponding values in *L. racemigerum*. If there were no other factors to be taken into account we should therefore expect the final size of 'Chinaman' to be correspondingly greater than that of *L. racemigerum*. It must be remembered, however, that there are growth rate differences between the two strains, and furthermore, that the dwarfness of 'Chinaman' is of a peculiar kind, being caused chiefly by rapid falling off in the growth rate of the stem after the fifteenth week.

III. SUMMARY OF THE EXPERIMENTAL DATA

The data presented in the foregoing pages may now be summarized. A population of F_1 hybrid plants was grown side by side with populations of its tall parent (*L. racemigerum*), and its dwarf parent (*L. esculentum* var. 'Chinaman'), and the populations were sampled during the flowering period from the thirteenth to the twenty-third week after sowing. The hybrid was found to exceed both of its parents in the following respects:

(1) Total dry weight.
(2) Dry weight of stems.
(3) ,, ,, ,, leaves.
(4) The number of leaves.
(5) Total leaf area.
(6) The size of the individual leaves.
(7) Embryo weight.
(8) Size of the young shoot primordia.
(9) The number of laterals.

In the following respects, however, the hybrid did not differ significantly from its tall parent:

(1) Efficiency index measured as dry weight.
(2) Relative growth rate of stems measured as dry weight.
(3) ,, ,, ,, ,, leaves ,, ,, ,, ,,
(4) ,, ,, ,, measured as height.
(5) Differential growth ratio between stem and leaf weight.
(6) Relative rate of production of leaves.
(7) Length of the period of growth.
(8) Assimilation rate per unit area.
(9) Height.
(10) Mean internode length.
(11) Cell size in leaf epidermis.

IV. DISCUSSION

The manifestations of heterosis.

It is apparent from the data presented in this paper that the phenomenon of heterosis does not involve a general increase in vegetative vigour in the

F_1 generation, but is a specific effect which is manifest to different degrees in the various organs of the plant body. In the present experiment, for instance, the leaf weight of the hybrid exceeded that of the tall parent by over 115 per cent., the stem weight by 45 per cent., the mean leaf area by 94 per cent., and the leaf number by only 12 per cent. Increase in height, which is one of the most frequent manifestations of heterosis, was not shown at all by this particular hybrid; neither was there any increase in internode length in the F_1 generation.

The effects of heterosis vary, not only between different organs of the same hybrid, but also between different hybrids. Even in the hybrids resulting from crosses of different varieties of the same species, the amount of variation is considerable. Sometimes one particular character is affected and sometimes another, and the fact that such diversity exists renders it improbable that heterosis is nothing more than a 'stimulation' effect resulting from the union of the egg with a foreign sperm, and indicates that some genetical explanation is required. Before discussing possible genetical interpretations however, it is necessary to consider in some detail the more immediate causes of heterosis.

The causes of heterosis.

The final size of any organism depends on three factors, and differences in any one of these may be responsible for differences in final size. They are:

(i) The relative rate of growth, as determined by inherited and environmental factors.
(ii) The duration of the period of growth.
(iii) The initial size.

Applying these considerations to the case under discussion we find that there are only two possible ways of explaining the observed manifestations of heterosis. It has been shown in this paper that the hybrid does not differ from its tall parent, either in the relative growth rate of the whole plant, measured as increase in dry weight or as increase in height, or in the relative growth rates of the separate organs. Similar results have already been presented, both for maize and tomato, in the earlier papers of this series (Ashby, 1930, 1932, 1937). Hence, increased size in the F_1 generation must be due either to a longer period of growth or to a greater initial size.

By 'initial' size is meant here, not the size of the fertilized egg, but the size of the plant at the earliest sampling in the particular experiment under discussion. In the present series of experiments 'initial' size is taken as the size of the primordia in the resting embryo. Size-heterosis in the embryos of vigorous F_1 hybrids is now an established fact, and there is often a close correspondence between the amount of heterosis present in the embryo and that present in the mature plant. The disadvantage of total embryo weight as an exact measure of 'initial capital' however, especially in the case of the tomato, has already been discussed (p. 398). Nevertheless, it has been established beyond doubt in at least four instances (Ashby, 1930, 1932, 1937) that hybrid vigour is due to the maintenance, on a logarithmic scale, of an initial advantage in size of primordia, and the results of the experiment described in this paper are in agreement with this view.

Sprague (1936) has recently studied the growth rates of two pure lines of maize and their reciprocal F_1 hybrids, and claims to have established that during the early seedling stage the hybrids grew faster than either parent. This conclusion is based on inference and is not supported by his statistical analysis. The data for the first forty-five days of growth and for the entire growth period of eighty days were fitted to linear and curved regressions respectively. In neither case were there significant differences between the relative growth rates of the hybrids and their parents. Sprague states that 'hybrid embryos are in general heavier than selfed embryos' and draws from this the erroneous conclusion that the relative growth rate of hybrids exceeds that of inbred lines between fertilization and maturity of seed. Moreover, his own data show no significant differences in embryo weight between selfed lines and hybrid lines. Sprague's data do, however, establish the fact that heterosis in the mature plant need not be preceded by heterosis in the embryo, and in this respect they differ from the data published by Ashby (1930, 1932, 1937). The correct inference to be made from this fact is that the hybrids either germinate more rapidly or grow faster in the seedling stage. There is as yet no evidence as to which of these inferences is correct, though the writer has evidence that in one instance (see p. 382) the hybrids germinate more rapidly than either parent.

There are certain aspects of heterosis however, which call for an extension of this hypothesis. As examples might be quoted the greater number of leaves and lateral branches of the hybrid, such as was observed in this experiment, and the earlier flowering and increased productivity which frequently accompany heterosis. Such differences as these are attributable to the second of the size determining factors mentioned above, namely, to differences in the duration of the growth period. To quote again from the present series of experiments, it has been found (Ashby, 1937) that the increase in leaf number which accompanies heterosis is due to the fact that the unfolding of the leaves in the hybrid begins at a slightly earlier date than in the parent lines. In other words, the hybrid is at a slightly later stage of development than its parents at the time of sampling.

The genetical basis of heterosis.

These investigations, besides throwing light upon the more immediate causes of hybrid vigour, also yield information on the mode of inheritance of various physiological characters. The characters which have been investigated include the efficiency index, assimilation rate, respiration rate (Ashby, 1932), and the differential growth ratio between stem weight and leaf weight. All these characters show complete dominance, and in every case it has been found that the higher of the two parental rates is dominant over the lower.

These two conditions are precisely the ones which are postulated in Jones's 'dominance theory' of hybrid vigour. If then we cross a plant of the constitution *AA bb CC dd*, with a plant of the constitution *aa BB cc DD*, where *A B C D* are growth rate factors, we should obtain an F_1 hybrid of the constitution *Aa Bb Cc Dd*, which on the assumption of complete dominance would have a growth rate double that of its parents. A case such as this, where hybrid vigour can be attributed to the complementary action of dominant genes from the two parents, is provided by the well-known investigations of Keeble and Pellew (1910) on tall and dwarf peas, which is quoted by Jones (1917). Such cases, however, are rare, and for this reason Jones's hypothesis cannot be accepted as a universal explanation of heterosis.

The failure of the theory to explain the results of the present series of experiments is accounted for by the third condition postulated in Jones's paper, viz. that size is determined by a great many separate factors, and that these factors are distributed fairly evenly in the different chromosomes. The experimental data indicate that this is not true of the relative growth rate. During the course of experiments on the tomato it has been observed that differences in relative growth rate between varieties are comparatively rare. If the relative growth rate were the resultant of the action of a large number of factors distributed in different chromosomes, we should expect differences in this character between varieties and species to be of very frequent occurrence, and it is inconceivable that different combinations of factors in a number of varieties should have precisely the same effect and result in the same relative growth rate.

Heterosis in embryos.

The above discussion deals exclusively with the problem of heterosis in the post-germination period of the life-cycle. We have still to account for the presence of heterosis in the resting embryo. This is at present a matter for speculation, as there are practically no experimental data on the growth rates of immature embryos.

Any differences in size between an F_1 hybrid embryo and that of its female parent can only arise from two possible causes. Either there are differences in relative growth rate between the embryos, or there are differences in the lengths of their growth periods. 'Initial' size (i.e. the size of the zygote) is presumably the same whether the egg has been fertilized by a sperm carrying a similar set of genes or by a foreign sperm with a different set.

If the genes which control growth in the pre-germination period of the life-cycle are the same as those which control it in the post-germination period, it is evident that the increased size of hybrid embryos can only be attributed to a longer period of growth. It may be that the heterozygous zygote begins to divide slightly earlier than the homozygous one, and such a difference could well explain the observed difference in the final size of the embryos. Such an explanation, if substantiated by experiment, would lead us back to the original 'stimulus' theory of heterosis. It may well be, however, that the genes controlling the growth rate of the embryo are not the same as those controlling growth in the period after germination. If this is the case, it is quite conceivable that we have in the F_1 generation a higher relative rate of embryo growth than in either of the parent lines, brought about by the kind of mechanism proposed by Jones.

In conclusion, it may be remarked that these experiments in no way solve the problem of heterosis. They do indicate, however, that the primary cause of the phenomenon lies in that part of the life-cycle between fertilization and the setting of seed, and it is to this part of the life cycle that future investigation must be principally devoted.

V. SUMMARY

Populations of a tall species of Lycopersicum (*L. racemigerum*) and a dwarf variety of the tomato (*L. esculentum* var. 'Chinaman') were grown side by side with a population of F_1 hybrid plants (*L. rac.* × 'Chinaman') in a greenhouse. The experiment was laid out in three randomized blocks with twelve rows of three plants in each block.

The populations were sampled at intervals from the thirteenth to the twenty-third week after sowing, observations being taken of the height, leaf number, inflorescence number, leaf area, dry weight of leaves and stems, &c., of each plant in the sample. The experimental data were treated by the method of analysis of variance.

The F_1 plants exhibited very marked hybrid vigour manifested in an increase in the dry weight of both stems and leaves, in total leaf area, in the size of the individual leaves, the number of leaves and the number of laterals, but not by any increase in the mean internode length or in height.

The larger size of the leaves of the hybrid compared with those of the tall parent was shown to be due to differences in cell number and not to any increase in cell size.

No evidence was obtained of increased metabolic activity in the vigorous F_1 hybrid. The relative rate of growth, measured as increase in dry weight and as height, were the same in the hybrid and the tall parent. There were no differences in the relative rate of production of leaves, the assimilation rate, or in the duration of the period of growth.

The differential growth ratio between stem weight and leaf weight was found to be inherited in the F_1 generation in the manner of a simple dominant.

The F_1 embryos were found to be significantly heavier than those of either parent strain, and evidence was obtained that heterosis was already present in very young shoot primordia.

The experiment supports the view that heterosis is due to the maintenance, on a logarithmic scale, of an initial advantage in the size of primordia in the embryo.

The results of the experiment are not in agreement with the current genetical interpretation of heterosis, and the possible reasons for this discrepancy are discussed.

It is considered that the primary cause of heterosis lies in that part of the life-cycle between fertilization and the setting of seed.

I wish to record my indebtedness to Dr. E. Ashby for his advice and criticism throughout the course of the experimental work and during the preparation of this paper, and to Prof. M. Skene in whose department the work has been carried out. I also wish to thank Miss M. Ackroyd for help with the routine work of sampling, Mr. A. C. Fabergé for advice on the statistical treatment of the results, and the Colston Research Society for a grant in aid of the research.

VI. APPENDIX

Statistical Methods and Tables

The experiment was laid out in three blocks, and in each block there were twelve rows of three plants. Thus in all 108 plants were grown, but of these eighteen were not utilized, so that the actual number of plants sampled was ninety. Hence there were eighty-nine degrees of freedom distributed in the following manner:

Variance due to:	
Time	4 d.f.
Block	2 d.f.
Strain	2 d.f.
Interactions:	
Time × strain	8 d.f.
Strain × block	4 d.f.
Block × time	8 d.f.
Strain × block × time	16 d.f.
Error	45 d.f.
Total	89 d.f.

The forty-five degrees of freedom for error is the remainder after the appropriate number of degrees of freedom for time, block, strain, and interactions have been subtracted from the total. Of these forty-five degrees of freedom, one is accounted for by 'individuals', since at each sampling two plants of each strain were taken from each block. The remaining forty-four are accounted for by the interactions of individuals with time, strain, and block, as follows:

Individuals	1 d.f.
I. × strain	2 d.f.
I. × block	2 d.f.
I. × time	4 d.f.
I. × strain × block	4 d.f.
I. × strain × time	8 d.f.
I. × block × time	8 d.f.
I. × strain × block × time	16 d.f.
Total	45 d.f.

In Table XIV is given the main analysis of the data for total dry weight.

In the present experiment we are chiefly interested in the variance due to strain, which will give information as to any absolute size differences between the strains, and the variance due to the interaction of strain with time, which will indicate any differences in relative growth rate between the strains. In the analysis of total dry weight (Table XIV) both these variances are highly significant. Before any conclusions can be drawn, however, it is necessary to know how they arise. There are three possible sources of variance, namely, between the two parent strains (*A* and *B*), between one parent and the hybrid (*A* and *C*), and between the other parent and the hybrid (*B* and *C*). The sums of squares for Strain and Strain × Time were accordingly decomposed as follows:

TABLE XIV

Analysis of Variance of Logarithms of Total Dry Weight

Variance due to:	Sums of sqs.	d.f.	Mean square.	'*z*'.	1 per cent. pt. '*z*'.
Time	39·955100	4	9·988775	*3·1457*	0·6954
Block	0·144213	2	0·072106	0·6801	0·8423
Strain	2·595431	2	1·297715	*1·6252*	0·8423
Time × strain . . .	0·877441	8	0·109680	*0·8898*	0·5773
Strain × block . .	0·221349	4	0·055337	0·5478	0·6954
Block × time . . .	0·210077	8	0·026259	0·1749	0·5773
Time × strain × block .	0·394614	16	0·024663	0·1436	0·5224
Error	0·832519	45	0·018500		
Total	45·230744	89			

TABLE XV

Decomposition of Variances Due to Strain and to Interaction of Strain with Time

Variance due to:	Sums of sqs.	d.f.	Mean square.	'*z*'.	1 per cent. of '*z*'.
Strain:					
Between *A* and *B* .	0·339302	1	0·339302	*1·4545*	1·0116
,, *B* ,, *C* .	2·534637	1	2·534637	—	1·0116
,, *A* ,, *C* .	1·019207	1	1·019207	—	1·0116
Strain × time:					
Between *A* and *B* .	0·585923	4	0·146480	*1·0345*	0·6954
,, *B* ,, *C* .	0·675836	4	0·168959	*1·1059*	0·6954
,, *A* ,, *C* .	0·054405	4	0·013601	—	0·6954

It must be emphasized that the three sets into which the variances due to strain and the interaction of strain and time have been partitioned are not orthogonal, because when any two are fixed the third is determined. This explains why the original two degrees of freedom for strain variance on decomposition yielded three degrees of freedom. It is not legitimate therefore, to compare these three degrees of freedom with one another. We can, however, consider each one separately and carry out the ordinary '*z*' test, as has been done in Table XV. The interpretation of the results of the analysis has already been discussed (p. 387).

Having established the significance of the variance due to strain, both between the two parents and between the parents and the hybrid, we require to know whether this variance is limited to any particular period of time, or whether it is distributed evenly over the duration of the experiment. This information is given by the further decomposition of strain variance presented in Table XVI.

TABLE XVI

Further Decomposition of Variance Due to Strain

Variance due to:	Sums of sqs.	d.f.	Mean square.	‘*z*’.	1 per cent. pt. ‘*z*’.
Strain between *A* and *B*:					
13th to 15th week	0·000003	1	0·000003	—	—
15th ,, 17th ,,	0·004003	1	0·004003	—	—
17th ,, 19th ,,	0·005070	1	0·005070	—	—
19th ,, 23rd ,,	0·256697	1	0·256697	*1·3151*	1·0116
Between *A* and *C*:					
13th to 15th week	0·238077	1	0·238077	*1·2775*	1·0116
15th ,, 17th ,,	0·413701	1	0·413701	*1·5536*	1·0116
17th ,, 19th ,,	0·612493	1	0·612493	*1·7499*	1·0116
19th ,, 23rd ,,	0·440649	1	0·440649	*1·5852*	1·0116

We see from this analysis that the two parent strains do not differ significantly in dry weight until after the nineteenth week, whereas the hybrid and the tall parent differ significantly throughout the experiment, from the thirteenth week onwards.

TABLE XVII

Analysis of Variance of Logarithms of Leaf Number

Variance due to:	Sums of sqs.	d.f.	Mean square.	‘*z*’.	1 per cent. pt. ‘*z*’.	5 per cent. pt. ‘*z*’.
Strain:						
Between *A* and *B*	0·421849	1	0·421849	*1·8085*	1·0116	0·7141
,, *B* ,, *C*	0·763656	1	0·763656	*2·1052*	1·0116	0·7141
,, *A* ,, *C*	0·050344	1	0·050344	*0·7456*	1·0116	0·7141
Total =			1·235849			
Total strain variance		2	0·411949	*1·7966*	0·8423	—
Strain × time						
Between *A* and *B*	0·316319	4	0·079079	*0·9714*	0·6954	—
,, *B* ,, *C*	0·332024	4	0·083006	*0·9956*	0·6954	—
,, *A* ,, *C*	0·005356	4	0·001314	—	—	—
Total =			0·163399			
Total strain × time var.		8	0·054466	*1·5698*	0·5773	—
Error	0·509943	45	0·011332			—

The analysis of the data for leaf number was carried out along somewhat different lines, a shorter form of analysis being used. As it had already been demonstrated that the variance due to Blocks was not significant, this variance was not calculated in the analysis of leaf number; neither were the variances due to Time, and interactions of Time and Block and of Block and Strain. The variances due to Strain between *A* and *B*, between *B* and *C*, and between *A* and *C*, were first calculated, and the mean of these three variances gave the total Strain variance. A similar procedure was adopted in the calculation of the variance due to the interaction of Strain and Time. The analysis is given in Table XVII.

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EXPLANATION OF PLATE XVIII

Illustrating Mr. L. C. Luckwill's paper on: 'Studies in the Inheritance of Physiological Characters. IV. Hybrid Vigour in the Tomato. Part 2.'

All the photographs were taken twenty-six weeks after sowing.

Fig. 1. *Lycopersicum esculentum* var. 'Chinaman'. Note fasciated fruits, 'wilty' foliage, and dwarf habit.

Fig. 2. As for fig. 1.

Fig. 3. *Lycopersicum racemigerum* (on left) and an F_1 hybrid plant. Note the large size of the leaves of the hybrid and the absence of height heterosis.

Fig. 4. In the foreground is the dwarf parent (*L. esculentum* var. 'Chinaman'). The gardener is tying up a hybrid plant and behind him is the tall parent (*L. racemigerum*).

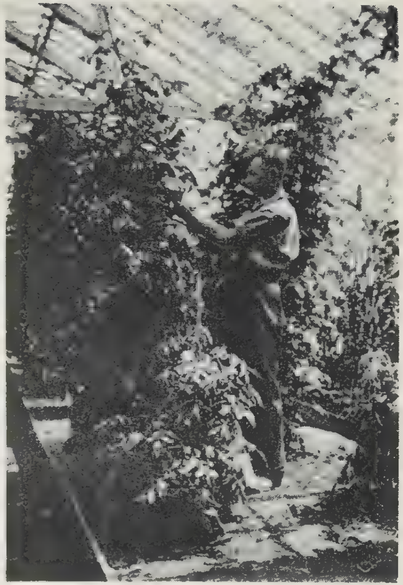
Fig. 5. *Lycopersicum racemigerum.*

Fig. 6. *L. racemigerum* × *L. esculentum* var. 'Chinaman'.

1

2

3



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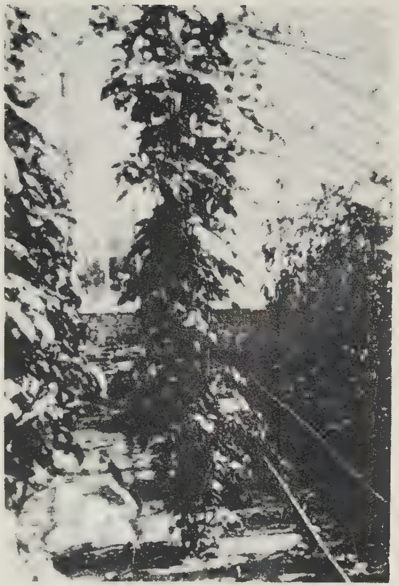

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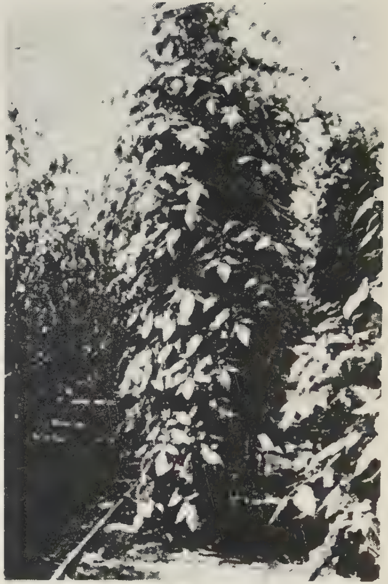

6

Huth. Stubbs X. Kent

LUCKWILL — HYBRID VIGOUR.

The Phylogenetic Value of Certain Anatomical Features of Dicotyledonous Woods

BY

L. CHALK, M.A., D.Phil.

(*Imperial Forestry Institute, Oxford*)

With one Figure in the Text

INTRODUCTION

IT has long been recognized that there is a close connexion between wood anatomy and floral development, and the wood anatomist is often consulted by the taxonomist as to the probable affinities of doubtful genera or families. There are, however, some serious discrepancies between current taxonomic classifications and wood anatomy, and it is important to determine where and why the relation between wood anatomy and taxonomy breaks down.

The structure of wood is probably more conservative than that of the flower, and it is not surprising to find that the minor floral differences by which many species are distinguished from one another are not reflected in the wood; thus the woods of different species of a genus are often indistinguishable. Differences between genera are usually clearly defined in the wood, and where they are absent it can often be shown that the taxonomic distinction is open to question.

Up to this point the agreement between wood anatomy and taxonomy is extremely close, and discrepancies can almost invariably be explained on re-examination of the data. In the composition of the families, however, agreement is not so close. Many families, for example the Sapotaceae, are evidently sound natural groups in which the genera are clearly linked together by important common characters, but others include such a mixture of types of wood that it is difficult to find any characters that are common to all the genera other than those that are common to all Dicotyledons. Very often these differences within a family can be related to taxonomic subdivisions; for example, in the Rosaceae the woods can be divided into two groups, one of which includes all the genera of the Chrysobalanoideae and the other those of the Pomoideae, Rosoideae, and Prunoideae. Provided that the sub-families are really related, this only means that some sub-families should probably rank as families. Sometimes, however, as in the Rosaceae, the different types of wood found within a family bear little relation to each other, and it would be no better to have them grouped together as two families of an order than as two sections of a family. In some families there are marked differences in the type of wood

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in different sections and the proximity of these types as members of the same family or order will tend to obscure the relation between wood anatomy and taxonomy. On the whole, however, the grouping of genera into families is supported by the anatomy of their woods, but in the relations between the families and in the grouping of the families into orders, the parallel between wood anatomy often disappears completely. Though there are some orders, for example the Ebenales, in which the wood anatomy is fairly uniform throughout, most of the large orders include such varied types of wood that they appear to be quite meaningless as groups. This, perhaps, accounts for the fact that for the taxonomist it is the family that counts, and that the orders are often very difficult to define and of little practical importance.

It is possible that there is no relation between wood anatomy and floral development in these larger groups. But as the relation can be shown to exist in both the smaller units such as genera and in the very large groups such as the gymnosperms and angiosperms, and Monocotyledons and Dicotyledons, it is difficult to believe that it is really lacking in between. It seems much more probable that the apparent lack of relation is due to misinterpretation of the facts, and it is perhaps significant that taxonomic botanists are by no means unanimous as to the relationships between families or the composition of the orders. But though this may suggest that it is the taxonomic grouping that is at fault, the same result might be produced by a faulty interpretation of wood anatomy. It is therefore essential to consider carefully the significance of the particular features in wood that can be used for this purpose.

In considering the phylogenetic value of wood characters one possible source of doubt is that the elements appear to have followed the same general lines of specialization in all dicotyledonous woods. For example, specialization in the vessel has been shown by Frost (1930) to be from a type with scalariform perforation plates and pitting to one in which the perforation plates are simple and the pitting alternate. Since probably all dicotyledonous woods have passed, or are capable of passing, through these stages, these features indicate degree of specialization rather than relationship, and may be misleading if used to demonstrate phylogenetic affinity.

It must also be recognized that taxonomy has played a large part in the proof of these series of development in elements of the wood, and the reason for regarding a feature in the wood as unspecialized is usually that it is characteristic of plants that the taxonomist regards as unspecialized. Consequently it is in the nature of a vicious circle to use one of these features to prove or disprove the validity of any taxonomic grouping. The case for regarding scalariform perforation plates and bordered pits in the fibrous elements as unspecialized features is, however, partly based on their occurrence in the wood of ferns and gymnosperms respectively, so that the direction of specialization in these elements must be assumed to be correctly interpreted as long as it is agreed that ferns and gymnosperms are less advanced than

Dicotyledons. The first part of this investigation has been devoted to showing that there are several features that can be used to indicate the degree of specialization and that the evidence of each feature confirms that of the others, so that the whole forms a body of evidence that cannot be the result of chance.

In the second part of the investigation these wood characters indicating degree of specialization have been used to test and compare the sequences adopted in three different taxonomic systems. It was found that none of these sequences receives very much support from the evidence obtainable from wood anatomy. For example, the wood anatomy of the Polypetalae of Bentham and Hooker (1862–83) is on the average rather more highly specialized than that of their Monochlamydeae, and the Metachlamydeae (Sympetalae) of Engler (1924) contains a high proportion of primitive woods. The sequence adopted by Hutchinson (1926) for the families in the Archichlamydeae is better supported by the evidence obtainable from the wood anatomy than that of either of the other two systems examined.

THE ANATOMICAL FEATURES USED IN THE INVESTIGATION

In order to make the survey as comprehensive as possible all the available specimens were used irrespective of whether they were authenticated by herbarium material or not. The great majority of the wood specimens examined have, however, been named from herbarium material collected from the same tree, and very few of the genera are not represented by at least one authentic specimen. Unauthentic material was discarded if it showed any marked differences from closely allied authentic species. In spite of this a few wrongly named specimens may have been included, but these should not seriously affect the conclusions drawn, and, provided they have not been assigned to the wrong family, they should not affect the results at all.

The genus was used as the basis for numerical comparison as it forms a comparatively homogeneous unit, and if a feature is present in the wood of one species of a genus, it will usually be present in all. The interpretation of generic rank is probably much more consistent among taxonomic botanists than is the concept of a species, and the number of genera in which any particular anatomical character occurs is probably much more significant than the number of species; particularly as the latter is often dependent to some extent on whether the genus has been intensively studied, and by whom. The desirability of using the genus rather than the species as the unit of classification has been referred to by Rendle (1936).

The following features have been used for this investigation: fibres, parenchyma, vessel perforation plates, and storied structure. The extreme forms of fibre-tracheids and libriform wood fibres are easily distinguished; but, as there is a complete series of gradations between the two types, the interpretation of the intermediate forms is largely a matter of personal choice. Reinders (1935) has suggested a rather complicated definition of the fibre-tracheid based on the work of Janssonius, but this has been adversely criticized by Bailey (1936). For the purpose of this investigation the definition of a fibre-tracheid has been based on the sectional view of the pit, and the presence of a pit-chamber that can be seen without using magnification greater than 500 times. The definition is an artificial one and does not by any means remove the personal factor, but it has the merit that it can be used rapidly and consistently.

Septate fibres presented no particular difficulty apart from the usual one of distinguishing between septa and fungal hyphae.

The types of parenchyma suggested by the International Association of Wood Anatomists (1933) are not entirely suitable for the present investigation. Bailey (1917) has drawn attention to the change that has taken place in the meaning attached to 'metatracheal' parenchyma; originally used by Sanio for parenchyma linking vessels together (corresponding to the 'confluent' type of the International Association of Wood Anatomists (1933)), the term is now used for parenchyma that is independent of the vessels. 'Metatracheal' in this sense, however, does not include scattered parenchyma cells, for which the term 'diffuse' is retained. These two types, metatracheal and diffuse, grade into one another so that there is no clear line of demarcation between them, and it would be very convenient to have a single term to include both of them. The term 'apotracheal' parenchyma will be used for this purpose, implying that the position of the parenchyma is not determined by that of the vessels, as is the case with paratracheal parenchyma, though parenchyma cells must often touch the vessels, particularly where the lines of parenchyma are numerous.

As the investigation proceeded, the difference between 'apotracheal' parenchyma and the types associated with the vessels (paratracheal) appeared to be the most fundamentally important distinction that could be drawn, provided that a distinction could be made between broad bands that are apotracheal in origin and similar bands that are essentially paratracheal in origin. Specialization of both apotracheal and paratracheal parenchyma seems to lead ultimately to the same result, i.e. broad bands. Apotracheal bands are sometimes either so broad or so close together that practically all the vessels are touched by them, and the difference from confluent paratracheal bands is often obscure. In the majority of cases, however, they can be distinguished by examining areas where the parenchyma is less abundant, for example, the early part of the growth ring. It will be found that in such areas the parenchyma, if apotracheal, will still consist of bands, though these will be farther apart and more obviously independent of the vessels, but if the bands are paratracheal in origin, in areas where the parenchyma is less abundant it will tend to be aliform, rather than confluent, and to be obviously associated with the vessels. This distinction is difficult to apply in some cases, but it appears to have a real phylogenetic significance and to correspond to taxonomic classification. For example, in the Papilionaceae there are many species with bands of parenchyma that are described by some authors as metatracheal [for example, *Pterocarpus dalbergioides*, in Pearson and Brown (1932)], though on the lines indicated above they can be shown to be paratracheal. By interpreting parenchyma in this way it is possible to show that many families that otherwise appear to be 'mixed' in regard to their parenchyma are in reality homogeneous.

In defining storied structure, no distinction has been drawn between woods in which all the elements are arranged in stories and those in which only some are so organized. There is a complete series of gradations from non-storied to distinctly storied arrangement, and any line between them is bound to be largely a matter of personal judgement. But, as in the case of fibre-tracheids and libriform fibres, consistency was of more importance than the actual limits set to storied structure. Every effort was therefore made to apply consistently the standard adopted. Any genus in which storied arrangement occurred in some species was counted as having storied arrangement.

In investigating scalariform perforation plates, a distinction was originally drawn between woods in which all the perforation plates are scalariform and those in which scalariform and simple plates occur side by side. But it was found more satisfactory to group them together owing to the comparatively small number of genera involved. If scalariform perforation plates were consistently present in any species the genus was considered as having this feature. Where scalariform perforation plates occurred only very occasionally in some samples (for example *Castanea sativa*) they were disregarded. The foraminate types of multiperforate plates that occur, for example, in the Bignoniaceae have been shown (Chalk, 1933) to be different in significance and therefore have not been considered as evidence of an unspecialized condition.

Scalariform intervascular pitting was originally used as an unspecialized feature, but was discarded owing to the large proportion of unspecialized woods that have solitary vessels and therefore no intervascular pits.

THE SIGNIFICANCE OF THE ANATOMICAL FEATURES

There can be little doubt that scalariform perforation plates in vessels are an unspecialized feature, and it appears to be generally accepted that very short cambial initials represent a high degree of specialization. As it has been shown (Chalk and Chattaway, 1935) that short cambial initials are associated with storied structure, the latter feature can be used as an indication of a high degree of specialization. The fact that these two features—scalariform perforation plates and storied structure—have not been observed in the same genus shows that they do indeed represent widely separated extremes.

Using these two features it is possible to divide the genera of the Dicotyledons into three groups representing different degrees of specialization. Group I with woods with scalariform perforation plates represents the least specialized group; Group II with woods with neither scalariform perforation plates nor storied structure is intermediate; and Group III with woods with storied structure is the most highly specialized. The middle group is by far the largest and includes approximately 70 per cent. of the genera.

This grouping of the genera helps to interpret the significance of other features in the wood. For example, the following table shows how the different types of fibrous elements are distributed in the groups.

TABLE I

Distribution of Types of Fibre

	Group I.	Group II.	Group III.	Average.
Total number of genera	154	876	231	—
Percentage of genera with:				
fibre-tracheids	52·5	29	6	27
libriform fibres	28·0	51	88	55
septate fibres	19·5	20	6	18

TABLE II

Distribution of Types of Parenchyma

	Group I.	Group II.	Group III.	Average.
Percentage of genera with:				
Apotracheal parenchyma	53·5	41	36	42
Paratracheal parenchyma	30·5	53	80	55
Both types of parenchyma	0·0	4	18	6
Neither type of parenchyma	18·0	10	2	9

It will be seen that the figures in the table support the view that fibre-tracheids are a less advanced type of cell than libriform wood fibres (Bailey, 1917 and 1936). The percentage of genera with fibre-tracheids drops from over 50 in Group I to 6 in Group III, while the percentage of libriform fibres increases from 28 in Group I to 88 in Group III. The figures for septate fibres are of interest, as there appears to be no clear idea as to their origin or significance. It will be seen that they must be regarded as a primitive rather than an advanced type of element, as the percentage drops from 19·5 in Group I to 6 in Group III. But the middle group in this case contains the same proportion as Group I instead of being intermediate. They are, therefore, more or less on a level with fibre-tracheids and do not appear to form part of the series tracheid—fibre-tracheid—libriform fibre.

The same procedure has been adopted for types of parenchyma. The chief difficulty here, as has been pointed out in a previous section, lies in the classification of the two types, and it is of interest to see whether the grouping suggested gives any consistent results. The figures for the relative proportions of each type in these groups are given in Table II.

Here again there are distinct and consistent changes in the percentages from Group I to Group III, and these support the view that paratracheal distribution of parenchyma is associated with an advanced systematic position. For example, the percentage of genera with paratracheal parenchyma increases from 30 per cent. in Group I to 80 per cent. in Group III. The percentage of genera with apotracheal parenchyma, on the other hand, decreases from Group I to Group III, though the difference is less marked, the percentage falling from 53·5 in Group I to 36 in Group III.

Paratracheal parenchyma appears therefore to be more characteristic of specialized woods and apotracheal parenchyma of unspecialized genera. On the other hand, roughly a third of the genera in the lowest group (I) have paratracheal parenchyma, and a third of those in the highest group (III) have apotracheal parenchyma, so that paratracheal parenchyma, although it may be regarded as having been more successful biologically, does not in itself indicate a highly specialized condition. It seems probable that both types of parenchyma have comparatively remote origins and represent independent lines of development. If this is so, these two characters should prove very useful for demonstrating affinity (or the reverse) between large groups such as families.

The occurrence of both types of parenchyma in the same wood is comparatively rare, and only becomes at all common in the most advanced group.

In a few woods (about 12 per cent. of the whole) neither apotracheal nor paratracheal parenchyma was present. These woods can be divided into two sections, those with terminal parenchyma and those from which parenchyma is entirely absent. The number of genera in these groups, however, is very small (22 and 70 respectively) and no very definite conclusions can be drawn. It seems probable, however, that neither the absence of parenchyma nor the presence of terminal parenchyma only is an advanced character.

The Relation between Wood Anatomy and Systematic Classification

It has been shown that three independent characters (scalariform perforation plates, fibre-tracheids, and storied structure) can be used to separate dicotyledonous woods into specialized and unspecialized groups, and that the results obtained with each character confirm those obtained with the other two. The same method has been used to determine whether the sequence adopted in any floral classification is confirmed by the wood anatomy. Three systems of classification have been analysed in this way, those of Bentham and Hooker (1862–83), Engler (1924), and Hutchinson (1926), and these will be discussed separately.

The procedure adopted for each was to divide the orders of the Dicotyledons into three groups, Group I including the earlier or less advanced orders and Group III the later or most advanced orders.

If a systematic sequence has any phylogenetic significance, the lowest group (I) should contain a higher proportion of genera with scalariform perforation plates than the intermediate group (II), and this feature should be relatively least common in the highest group (III). Similarly, Group I should have the highest proportion of genera with fibre-tracheids and the lowest proportion with storied structure.

The differences between the groups are often very slight and it is necessary to have some standard by which to judge whether any particular figure differs significantly from the average, or is within the probable limits of chance variation. For this purpose the χ^2 method has been used,[1] and the values for χ^2 and for P [obtained from Fisher's table (1928)] have been included in the tables. Any value of P less than 0·01 indicates that the figure differs significantly from the average.

Bentham and Hooker's classification.

In this system there are already three suitable groups, the Polypetalae (Group I), the Gamopetalae (Group II), and the Monochlamydeae (Group III). For each of these it has been calculated what percentage of genera have (1) scalariform perforation plates, (2) fibre-tracheids, and (3) storied structure, and in Table III the three systematic groups (I, II, and III) are shown in ascending order of specialization according to each of these features.

TABLE III

Distribution of Wood Characters in Bentham and Hooker's System of Classification

	Sequence.	Genera						
		No.	%	χ^2_a	P_a	χ^2_b	P_b	
Scalariform perforation plates	↑ II	15	3	11·3		14·2	<0·01	Significant
	I	110	14	0·7		1·9	0·18	Not significant
	III	41	17	3·0		4·7	0·03	Barely significant
				15·0	<0·01			
Fibre-tracheids	↑ I	177	21	11·2		29·4	<0·01	Significant
	III	61	25	1·9		2·5	0·12	Not significant
	II	119	47	56·2		71·0	<0·01	Significant
				69·3	<0·01			
Storied structure	↑ I	183	24	15·3		39·3	<0·01	Significant
	II	34	14	3·8		4·6	0·03	Barely significant
	III	13	6	25·8		31·4	<0·01	Significant
				44·6	<0·01			

I = Polypetalae, II = Gamopetalae, III = Monochlamydeae.

It will be seen that the sequence of the groups differs for each feature and that any of the groups may be regarded as specialized or unspecialized according to which wood character is selected. For example, Group II, the Gamopetalae, is at the top of the sequence if judged by the proportion of genera with scalariform perforation plates, but is the most primitive group if judged by fibre-tracheids. The other two groups also can be interpretated differently according to which character is used, and the wood anatomy can be said to confirm the usual assumption that the main divisions of the Dicotyledons in this system have little phylogenetic significance.

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[1] The total χ^2_a, or rather the probability P_a of its occurrence with two degrees of freedom, uses the probability that the given distribution could be obtained by chance from an entirely homogeneous population of genera. The contributions to the total χ^2_a of each group are also shown and give a rough indication of which group differs most from the average. A more precise statement of the discrepancy of each group from the average and of the probability of such a discrepancy is given in the columns χ^2_b and P_b, χ^2_b being calculated from a 2 × 2 table for the group in question as compared with the remaining two groups, and P_b being the probability of the occurrence of such a χ^2 (taken with one degree of freedom).

The principal objection to regarding this system as a phylogenetic one seems to be that it separates the Monochlamydeae (Apetalae) from the Polypetalae and assumes that apetaly is an advanced character. The latter view is certainly not borne out by the wood anatomy. Group III (Monochlamydeae) occurs at the beginning rather than at the end of the sequence, and in every case it is below Group I (Polypetalae). It is doubtful, however, whether the difference between the two groups is really significant for the proportion of genera with scalariform perforation plates.

But, though it can be shown that apetaly is usually associated with unspecialized woods, some apetalous families, such as the Moraceae, Nyctaginaceae, and Ulmaceae, have highly specialized woods, and it seems probable that apetaly may have a different significance in different groups of plants.

Engler's classification.

In the system of Engler (1924) the Gamopetalae of Bentham and Hooker become the Metachlamydeae (Sympetalae) and this group is regarded as the most advanced. The Polypetalae and Monochlamydeae are combined into one group, the Archichlamydeae, thus giving only two main groups. The sequence of the orders within the Archichlamydeae is, however, based on phylogenetic considerations, and it is therefore legitimate for the present purpose to subdivide it so as to give three main groups with the Metachlamydeae. This subdivision was made arbitrarily so as to include approximately equal numbers of genera in each group without splitting any order. The most convenient division from this point of view includes Orders 1 to 21 in Group I and Orders 23 to 30 in Group II. Order 22, the Pandales, was not represented. The distribution of scalariform perforation plates, fibre-tracheids, and storied structure in these groups is shown in Table IV.

Neither Group I (orders 1 to 21) nor Group II (orders 23 to 30) show any significant difference from the average as regards any of the anatomical features. It so happens, however, that this subdivision of the Archichlamydeae places the large family Leguminosae in Group I. Since many of the woods of this family are highly specialized (particularly those of the Papilionatae), their inclusion in the lowest group might mask the unspecialized character of the other woods of this group. Actually, however, it makes very little difference if the whole of the order containing the Leguminosae is transferred from Group I to Group II. The proportion of genera with fibre-tracheids remains the same at 27 per cent., that with scalariform perforation plates increases slightly to 17 per cent. and becomes just significantly above the average, and the percentage of genera with storied structure sinks to

6 per cent. These percentages are almost identical with those obtained for the Monochlamydeae of Bentham and Hooker.

The advancement of the Metachlamydeae (Sympetalae) to the position of the most highly specialized group is supported by the low percentage of genera with scalariform perforation plates, but the group should be the least advanced if judged by fibre-tracheids. The position in the sequence suggested by storied structure does not appear to be of very much significance.

TABLE IV

Distribution of Wood Characters in Engler's System of Classification

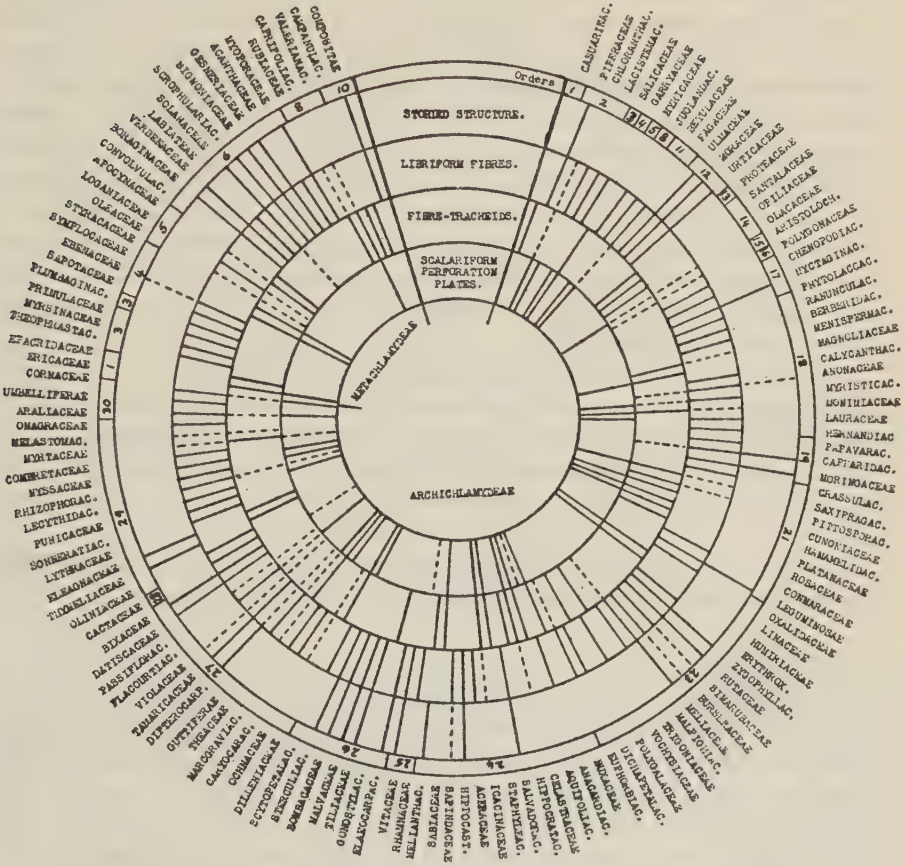
	Sequence.	Genera No.	%	χ^2_a	P_a	χ^2_b	P_b	
Scalariform perforation plates.	↑ III	15	6	8·5		11·0	<0·01	Significant
	II	85	13	1·2		2·5	0·12	Not significant
	I	53	14	0·8		1·2	0·27	,, ,,
				10·5	<0·01			
Fibre-tracheids	↑ II	130	20	16·6		2·6	0·11	Not significant
	I	104	27	0·1		0·3	0·60	,, ,,
	III	110	47	47·5		45·2	<0·01	Significant
				64·2	<0·01			
Storied structure.	↑ I	80	21	1·1		1·5	0·21	Not significant
	II	120	19	0·2		0·3	0·60	,, ,,
	III	34	14	3·8		5·0	0·03	Barely significant
				5·1	0·06			

I = Archichlamydeae, Orders 1 to 21.
II = ,, ,, 23 to 30.
III = Metachlamydeae.

Finally, it may be said that there is no evidence from the wood anatomy that the system of Engler has any greater phylogenetic significance than that of Bentham and Hooker.

Adverse criticism of the system of Engler is usually directed against the early position assigned to many of the apetalous families, and the assumption that they are more primitive than such families as the Ranunculaceae and the Magnoliaceae. The former contains too few woody members for comparison but, judged by the occurrence of scalariform perforation plates, fibre-tracheids, and length of cambial initial, there is very little to choose between the Magnoliaceae and some of the Fagales, and neither is as primitive as some of the genera of the Chloranthaceae, Dilleniaceae, and Theaceae. From the standpoint of wood anatomy the sequence adopted in this system is unsatisfactory throughout; many highly specialized woods are included in the early orders, and unspecialized woods occur as frequently in the higher orders as they do in the lower. This is shown diagrammatically on page 419.

In this diagram the four circles represent four degrees of specialization based on wood anatomy, the inner ring—woods with scalariform perforation plates—being the lowest, and the outermost ring—woods with storied structure—being the most advanced. The names of the families are arranged round the outside of the circles and the lines inside the circles show whether the feature is present in that family or not. A dotted line indicates that the feature is present in only a few genera. For fibres a dotted line has also been used to show that neither fibre-tracheids nor libriform wood fibres preponderate in a family.



Comparison of the sequence of families and orders in Engler's classification and the degree of specialization in the wood.

The most striking feature of this figure is that the families represented in the inner ring—those with the least specialized woods—are evenly distributed round the circle, and that there is no tendency for these lower types of wood to occur mainly in the lower orders. The families with storied structure are also evenly spaced throughout the orders, except for a big group in the Tubiflorae (Metachlamydeae No. 6).

The features used in this diagram indicate degree of specialization rather than relationship, but, if they are combined with the distribution of the parenchyma, some idea may be obtained as to whether a group is homogeneous or not. It is not proposed to discuss the orders in any very great detail, but the following points may be of interest.

Most of the orders that are well represented by wood specimens are extremely heterogeneous as regards the occurrence of scalariform perforation plates, fibre-tracheids, and storied structure. For example, the following orders are well represented in both the inner and outer circles of the diagram: Ranales, Rosales, Geraniales, Parietales, and Myrtaeflorae. But these features probably only represent degrees of specialization, and it is not to be expected that all the genera in these large orders will have reached the same level; but it is remarkable that the whole range of wood development should be included in each of these orders. The types of parenchyma are not shown in the diagram, but, if they are taken into consideration, these orders appear even more heterogeneous and it is difficult to believe that they represent any natural grouping of families.

There are, however, some orders that are more satisfactory. For example, the Urticales forms a very homogeneous group, which appears to be much more highly specialized than is suggested by its systematic position. The Malvales also is a homogeneous, highly specialized group, except for the Gonystylaceae and Elaeocarpaceae, which seem to be out of place in this order.

Consideration of the orders of the Metachlamydeae (Sympetalae) will be deferred until after Hutchinson's system has been discussed.

Hutchinson's classification.

Hutchinson follows Engler in dividing the Dicotyledons into two groups, the Archichlamydeae and the Metachlamydeae, and his system differs from Engler's mainly as regards the sequence of the orders in the Archichlamydeae. A similar procedure has therefore been adopted to obtain three main groups by subdividing the Archichlamydeae. The two sub-groups of the Archichlamydeae (Orders 1 to 34, and 35 to 59) differ in composition from the corresponding sub-groups of Engler's system.

Group III, the Metachlamydeae, is the same as in Engler's classification and needs no further comment. Group I, the first half of the Archichlamydeae (Orders 1 to 34), is the least advanced group as regards scalariform perforation plates and storied structure, and has a significantly higher proportion of fibre-tracheids than the average. Group II, the second half of the Archichlamydeae (Orders 35 to 59), is intermediate as regards scalariform perforation plates but has a significantly lower proportion of genera with this feature than the average for all three groups, and may therefore be regarded as rather advanced; it is the most advanced group as regards both fibre-tracheids and storied structure.

Hutchinson's arrangement provides groups that differ more significantly and consistently from the average than do either of the other two systems examined. The position of his least advanced families (Group I) in particular is in much closer accord with the wood anatomy than the corresponding groups from either Bentham and Hooker or Engler.

TABLE V

Distribution of Wood Characters in Hutchinson's System of Classification

	Sequence.	Genera						
		No.	%	χ^2_a	P_a	χ^2_b	P_b	
Scalariform perforation plates.	↑ III	15	6	9·2		11·5	<0·01	Significant
	II	60	9	5·7		12·3	<0·01	,,
	I	79	22	34·2		48·0	<0·01	,,
				49·1	<0·01			
Fibre-tracheids.	↑ II	89	14	63·3		132·6	<0·01	,,
	I	138	39	23·9		35·5	<0·01	,,
	III	119	47	49·9		53·0	<0·01	,,
				137·1	<0·01			
Storied structure.	↑ II	177	27	29·3		80·5	<0·01	Significant
	III	34	14	4·9		4·2	0·48	Not significant
	I	23	7	33·0		31·0	<0·01	Significant
				63·2	<0·01			

I = Archichlamydeae, Orders 1 to 34.
II = ,, ,, 35 to 59.
III = Metachlamydeae.

The Metachlamydeae.

It has been shown above that the Metachlamydeae, the higher division of the Dicotyledons, is highly specialized if judged by the relative scarcity of scalariform perforation plates, but unspecialized if judged by the abundance of genera with fibre-tracheids. This indeterminate character of the woods of the Metachlamydeae is due to the inclusion of a mixture of all types of wood, highly specialized and unspecialized, and with paratracheal or apotracheal parenchyma. This diversity in the wood anatomy suggests that union of the petals is a character that is common to several independent lines of development and does not indicate any relationship.

The woods of the Metachlamydeae fall naturally into two groups according to the type of parenchyma. One group, woods with apotracheal parenchyma, includes the Ebenales (4), part of the Contortae (5)—the Apocynaceae—, and the Rubiales (8), and the other group, woods with paratracheal parenchyma, includes the Primulales (2), the Plumbaginales (3), part of the Contortae (5)—the Oleaceae and the Loganiaceae,—the Tubiflorae (6), and the Campanulatae (10). In the Tubiflorae some of the genera of the Scrophulariaceae and Solanaceae have apotracheal parenchyma. This division of the Metachlamydeae into two parts according to the type of parenchyma separates most of the unspecialized woods from the specialized ones and the group with apotracheal parenchyma is at a much lower level of development than that with paratracheal parenchyma.

The Ericales (1), however, do not fit well with this arrangement; the woods of both the Ericaceae and the Epacridaceae are unspecialized, but the parenchyma is paratracheal in many of the genera of the Ericaceae. Burtt Davy (1935) has suggested that from the taxonomic point of view the Ericales should be included in the Metachlamydeae.

The relative proportions of the different features in each group (omitting the Ericales) are given in the following table. It will be seen that if the families with apotracheal parenchyma were excluded from the Metachlamydeae, this would then be a homogeneous and very highly specialized group.

TABLE VI

Relative Specialization in the Two Groups of the Metachlamydeae

Parenchyma.	Total no. of genera.	Percentage of genera with:			
		Fibre-tracheids.	Libriform fibres.	Scalariform perf. plates.	Storied structure.
Apotracheal	123	67	23	4	0·7
Paratracheal	113	19	59	0	28·0
Average for all Dicotyledons		27	55	11	18·0

The low level of development of the group with apotracheal parenchyma is shown by the high percentage of genera with fibre-tracheids and the low percentage of genera with libriform fibres and storied structure. The percentage of genera with scalariform perforation plates is below the average but is greater than that for the other group. The high level of development of the group with paratracheal parenchyma is shown by the low percentages of woods with fibre-tracheids and scalariform perforation plates and the high percentages of genera with libriform fibres and storied structure.

EVIDENCE FROM FOSSIL WOODS

Hamshaw Thomas (1934, 1936) has drawn attention to the lack of agreement between the evidence from fossil plants and the generally accepted ideas as to which floral characters indicate a primitive or an advanced plant. It is of interest therefore to see whether the fossil evidence is in any closer agreement with the three groups or degrees of specialization which have been described for woods. For this purpose the list of fossil dicotyledonous woods given by Edwards (1931) is very convenient. The fossils given in this list (excluding Pleistocene forms) have been arranged into three groups as follows: Group I, with scalariform perforation plates, Group II, with neither scalariform perforation plates nor storied structure, and Group III, with storied structure. The number falling into each group is shown in Table VII, and is compared with the number that would be expected if the percentage of fossils with that feature were the same as the percentage of modern genera.

The modern genus is unfortunately not strictly comparable with the 'form genus' to which fossil woods are ascribed. Some fossil botanists prefer to give a name, such as Dipterocarpoxylon, that signifies affinity with a family rather than a name suggesting a modern genus. Consequently the number of genera listed for the fossil Dicotyledons probably falls far short of the number really represented. As a check the comparison has also been made on the number of so-called species in each group. These 'species' probably represent no more than different specimens in a good many cases, but they serve to give some idea of the relative abundance of the different types of wood.

TABLE VII

Comparison of the Proportion of Specialized and Unspecialized Features in Fossil and Existing Woods

	Group I. (scal. perf.)	Group II.	Group III. (storied structure)
(*a*) *Genera*			
Number in fossil record.	17 (35 per cent.)	25 (51 per cent.)	7 (14 per cent.)
Number expected from modern distribution of features.	5 (11 per cent.)	35 (71 per cent.)	9 (18 per cent.)
Difference from expected number.	+12	−10	−2
(*b*) *Species*			
Number in fossil record.	90 (38 per cent.)	120 (50 per cent.)	28 (12 per cent.)
Number expected from modern distribution of features.	26 (11 per cent.)	169 (71 per cent.)	43 (18 per cent.)
Difference from expected number.	+64	−49	−15

It will be seen that it makes very little difference to the relative proportions of the fossils in each group whether these are counted by species or by genera. In both cases the proportion of fossils in the unspecialized group (I) is much higher than the average for existing genera. But it must be emphasized that there are unavoidable possibilities of error in the comparison. The disparity between the classification of fossil and existing woods has already been referred to. This, however, is probably not very serious as there is no reason to suppose that one type of wood will be affected more than another. A more serious difficulty is that the feature necessary for placing the fossil in the right group is not actually recorded but must be inferred from the name. In the majority of cases this can probably be done with a reasonable degree of accuracy, but where the name given is indicative of a family and the feature is only present in some genera of the family, it is impossible to do more than guess whether the fossil should be counted as having the feature or not. Such cases fortunately are not common, but must detract seriously from the reliability of the comparison. It is, however, satisfactory to find that this evidence tends to show that the characters regarded as unspecialized by the wood anatomist were relatively more common in the past.

SUMMARY

1. Out of a total of 1,272 genera of Dicotyledons studied 11 per cent. had scalariform perforation plates, 27 per cent. fibre-tracheids, 55 per cent. libriform wood fibres, 18 per cent. septate fibres, and 18 per cent. storied structure.

2. The proportion of genera with fibre-tracheids is high in the group of woods with scalariform perforation plates and low in the group with storied structure. Septate fibres are relatively more common in association with scalariform perforation plates than with storied structure.

3. Two main types of parenchyma (other than terminal) are distinguished: (1) associated with the vessels (paratracheal), and (2) independent of the vessels (for which the term 'apotracheal' is suggested). The former is relatively more common in woods with libriform fibres and storied structure, and the latter in woods with fibre-tracheids and scalariform perforation plates. It is suggested that these types represent separate series of development, but that both tend towards the same ultimate form in highly specialized woods.

4. The proportion of woods with scalariform perforation plates appears to be higher among the recorded fossil Dicotyledons than among modern genera.

5. The parallelism between wood anatomy and taxonomy tends to disappear in the taxonomic groups larger than the family. Evidence of the degree of specialization in the wood is used to compare the sequences adopted in three taxonomic systems—those of Bentham and Hooker, Engler, and Hutchinson. Hutchinson's arrangement of the Archichlamydeae agrees more closely with the evidence obtainable from wood anatomy than that of Engler. The Monochlamydeae of Bentham and Hooker appears to be slightly less highly specialized than the Polypetalae, but neither group differs very markedly from the average for all the Dicotyledons examined. The Metachlamydeae (Sympetalae) includes a mixture of specialized and unspecialized woods, but these can be separated by means of the type of parenchyma present. The woods of Engler's Primulales, Plumbaginales, Contortae (part), Tubiflorae, and Campanulatae have paratracheal parenchyma and constitute a very highly specialized group.

6. A list is given of the families arranged in groups according to the degree of specialization of their woods; the type of parenchyma present is also indicated.

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I am indebted to Dr. J. Burtt Davy and Dr. M. M. Chattaway of the Imperial Forestry Institute, Oxford, for criticism and advice, and to Dr. J. Marschak of the Institute of Statistics, Oxford, for help in analysing the data.

APPENDIX

A List of the Families Studied, Grouped according to the Specialization of their Woods

The families represented in the collection at the Imperial Forestry Institute have been divided into three groups representing different degrees of specialization in their woods; the degree of specialization has been judged by the presence or absence of scalariform perforation plates and storied structure. Group I represents the unspecialized class and includes all the families which possess some genera with scalariform perforation plates. Group III, which represents the most highly specialized class, includes all the families in which some genera have storied structure.

Within each group the families are separated into two columns according to whether the parenchyma is apotracheal or paratracheal (see earlier), as it is thought that these two types of parenchyma represent two independent lines of development. In some of the families the parenchyma is not all of the same type and different genera of a family may fall into different places in this classification. This is indicated by adding the word 'part' in brackets after the family. Brackets round the name of a family indicate that only a small fraction of the genera occur in that group. Some exceptional genera in otherwise homogeneous families have been ignored.

APOTRACHEAL PARENCHYMA. PARATRACHEAL PARENCHYMA

GROUP I. *With Scalariform Perforation Plates*

Aquifoliaceae
Betulaceae
Buxaceae
Caprifoliaceae
Celastraceae
Cornaceae
Cunoniaceae
Dilleniaceae
(Ericaceae)
Fagaceae
Flacourtiaceae (part)
Garryaceae
Hamamelidaceae
Icacinaceae (part)
Lacistemaceae
Lecythidaceae
Marcgraviaceae (part)
Monimiaceae (part)
Myricaceae
Nyssaceae
Olacaceae
Platanaceae
Punicaceae
Rhizophoraceae
Saxifragaceae

Araliaceae
Chloranthaceae
Ericaceae
Flacourtiaceae (part)
Guttiferae (part)
Icacinaceae (part)
Lauraceae
Monimiaceae (part)
Myristicaceae
Staphyleaceae

APOTRACHEAL PARENCHYMA	PARATRACHEAL PARENCHYMA
Scytopetalaceae	
Styracaceae	
Symplocaceae	
Theaceae	

Families in which some genera are without apotracheal or paratracheal parenchyma: Buxaceae, Celastraceae, Ericaceae, Flacourtiaceae, Magnoliaceae, Monimiaceae.

GROUP 2. *With Neither Scalariform Perforation Plates nor Storied Structure*

Anonaceae	Acanthaceae
Apocynaceae	Anacardiaceae
Aristolochiaceae (part)	Aristolochiaceae (part)
Caryocaraceae	Burseraceae
Casuarinaceae	Calycanthaceae
Connaraceae	Campanulaceae
Convolvulaceae	Chenopodiaceae
Dichapetalaceae	Combretaceae
Ebenaceae	Crassulaceae
Epacridaceae	Gonystylaceae
Erythroxylaceae	Hernandiaceae
Euphorbiaceae	Hippocrataceae
Guttiferae (part)	(Lecythidaceae)
Juglandaceae	Loganiaceae
Linaceae (part)	Lythraceae
Melastomaceae (part)	Malpighiaceae
Menispermaceae	Marcgraviaceae (part)
Ochnaceae (part)	Melastomaceae (part)
(Oleaceae)	Mimosaceae
Opiliaceae	Myrsinaceae
Passifloraceae	Nyctaginaceae
Polygalaceae (part)	Ochnaceae (part)
Polygonaceae (part)	Oleaceae
Rosaceae	Oxalidaceae
Rubiaceae	Papavaraceae
Santalaceae	Phytolaccaceae
Sapotaceae	Pittosporaceae
(Scrophulariaceae)	Plumbaginaceae
Solanaceae (part)	Polygalaceae (part)
Sonneratiaceae (part)	Polygonaceae (part)
Trigoniaceae	Proteaceae
Valerianaceae	Ranunculaceae
	Rhamnaceae
	Rutaceae
	Sapindaceae
	Solanaceae (part)
	Sonneratiaceae (part)
	Theophrastaceae
	Umbelliferae
	Vitaceae
	Vochysiaceae

Families in which some genera are without apotracheal or paratracheal parenchyma: Aceraceae, Berberidaceae, Cactaceae, Onagraceae, Plantaginaceae, Primulaceae, Salicaceae, Scrophulariaceae, Theophrastaceae.

Families in which both apotracheal and paratracheal parenchyma are present in the same wood: Myrtaceae, Oliniaceae.

GROUP 3. *With Storied Structure*

APOTRACHEAL PARENCHYMA	PARATRACHEAL PARENCHYMA
Bixaceae	Bignoniaceae
Bombacaceae (part)	Bombacaceae (part)
Boraginaceae (part)	Boraginaceae (part)
Eleagnaceae	Caesalpinaceae
Eupomatiaceae	Capparidaceae (part)
Humiriaceae	Compositae
Malvaceae (part)	Datiscaceae
Sterculiaceae (part)	(Dipterocarpaceae)
Tiliaceae (part)	Elaeocarpaceae
Zygophyllaceae (part)	Labiatae
	Linaceae (part)
	Malvaceae (part)
	Meliaceae
	Melianthaceae
	(Mimosaceae)
	Moraceae
	Moringaceae
	Myoporaceae
	Papilionaceae
	Piperaceae
	(Rhamnaceae)
	(Rutaceae)
	Salvadoraceae
	(Sapindaceae)
	(Scrophulariaceae)
	Simarubaceae
	Sterculiaceae (part)
	Tamaricaceae
	Thymeleaceae
	Tiliaceae (part)
	Ulmaceae
	Urticaceae
	Verbenaceae
	Violaceae
	(Vitaceae)
	Zygophyllaceae (part)

Families in which some genera are without apotracheal or paratracheal parenchyma: Gesneriaceae, Hippocastanaceae.

Families in which both apotracheal and paratracheal parenchyma are present in the same wood: Bombacaceae, Dipterocarpaceae, Sterculiaceae, Tiliaceae.

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On the Primary Groups of Dicotyledons

BY

J. BURTT DAVY

IN teaching the systematic botany of tropical plants the writer has found the value of a system of classification in which the families are arranged in series of named groups, providing 'pigeon-holes' or compartments in which the mind can 'file' the names and characters of associated families, indicating, at the same time, their morphological development. Engler (1912) in his Syllabus has 41 'reihe' and 241 'familien' of Dicotyledons, while Hutchinson (1926) increases the corresponding orders to 76 and the families to 264; it is difficult for any, except perhaps those constantly employed in large herbaria, to remember the affinities of the particular families or even of the orders in such long and almost unbroken lines.

The arrangement of Bentham and Hooker (1862–83) has proved a useful medium for the training of systematic botanists, enabling students to acquire a sound knowledge of the families of Spermaphyta. This has been due in large measure to the breaking up of the list of 165 dicotyledonous families (there called 'orders') into named groups, i.e. 37 orders (there called 'cohorts'), collected into higher groups ('series'), and these into major groups ('divisions'). The delimitation of such groups, provided they are of convenient size and duly labelled, assists the mind to associate a family with its allies. With the additional material and information available to-day, it is obvious that some of the Bentham and Hooker groups could be subdivided or rearranged to advantage.

More than this is needed, however, and it is important that the lines of developmental trend should be reflected in the system of classification. How far it will be possible to combine the two desiderata before much further evidence is available has yet to be demonstrated.

For some years past the writer has been engaged on a comparative morphological study of the families of Spermaphyta, with a view to a possible regrouping on the lines indicated. In this he has been working in collaboration with Dr. Chalk and Dr. Chattaway, and has found that their studies in wood anatomy have thrown valuable light on phylogenetic relationships in cases where these have been masked by morphological modifications.

The publication of Dr. Chalk's paper in the present issue of the 'Annals' (pp. 409 to 428) makes it desirable to put forward some tentative views on a possible rearrangement based on floral structure, even though final conclusions have not yet been reached about the proper position of various anomalous families.

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Dr. Chalk has studied a large number of genera, widely distributed among many families ranging from the Magnoliaceae to the Compositae. These he has classified into three major groups, 'representing different degrees of specialization in their woods; the degree of specialization has been judged by the presence or absence of scalariform perforation plates and storied structure'.[1] It is satisfactory to find that the relative absence of specialization in the wood-structure of various families is correlated with relative primitiveness in their floral structure. Occasional marked exceptions occur, e.g. in the group of choripetalous tetracyclic Oligostemonae, which are definitely advanced in floral morphology while retaining primitive characters in the wood; such cases suggest interesting developments on further study.

In Group I of Dr. Chalk's series the wood-structure is unspecialized; this group includes all those families in which at least some of the genera have scalariform perforation plates. These families belong to the following, among other, orders: Fagales, Garryales, Myricales, Magnoliales, Laurales, Piperales, Dilleniales, Saxifragales (+Cunoniaceae), Hamamelidales, Theales, Ericales, Myrtales, and Hypericales; all these belong to the first (i.e. the least specialized) groups of the arrangement here proposed on the basis of floral structure.

Dr. Chalk's third group is the most highly specialized; it includes all those families in which at least some genera have storied structure. These families belong to the following, among other, orders: Tiliales, Malvales, Urticales, Meliales, Thymelaeales, Asterales, Personales, Boraginales, and Lamiales. These orders are morphologically advanced; the Tiliales, Malvales, and Urticales are the most primitive of them, but represent the most advanced in their particular morphological subclass (the Polystemonae). The remaining six orders belong to the subclass Oligostemonae, which is morphologically more specialized, and the four last-named are morphologically the most specialized of the Oligostemonae.

Group II of Dr. Chalk's arrangement is intermediate in character, comprising those families which have neither scalariform perforation plates nor storied structure. Morphologically these families occupy an intermediate position between the relatively primitive and relatively advanced families of Dicotyledons.

In Group II there occur, as would be expected, some members of orders found also in Group I on the one side, and in Group III on the other.

It is encouraging to find support in the wood anatomy for the provisional placing, on morphological grounds, of certain families whose affinities have been in some doubt, e.g.:

Aristolochiaceae in Group II instead of Group I.

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[1] *Perforation plate*: 'A term of convenience for the area of the wall (originally imperforate) involved in the coalescence of two members of a vessel.'

Scalariform perforation plate: 'A plate with multiple perforations elongated and parallel.'

Storied cambium: 'Cambium characterized by a horizontal seriation of the initials.'

Canellaceae ('Winteranaceae') and Dilleniaceae in Group I instead of Group III.

Ebenales in Group II instead of Group III.

The differences between the several 'natural' systems (as opposed to purely artificial systems) are largely due to differences in concept of the relative primitiveness or relative development of the several characters used as the basis of the particular classification. Some light is thrown on the question of relative value of characters by comparative morphology, ontogeny, anatomy, and palaeontology; in the final analysis it may become a question of trial and error whether the use of certain characters brings together families which are obviously allied, or whether it places them far apart.

Characters may be considered in pairs of opposites; the absence of a feature is—in this sense—a character, as much as its presence.

In the preparation of the classification here suggested, the following characters have been used as guides to relative primitiveness, their opposites indicating progressive development:

Relatively primitive.	Relatively advanced.
Apocarpy	Syncarpy
Pluricarpellary gynaeceum	Monocarpellary gynaeceum
Polystemony	Oligostemony
Spiral arrangement of floral-leaves	Cyclic arrangement of floral-leaves
Polyphylly of the perianth (the number of leaves indefinite and often undifferentiated)	Perianth leaves definite and differentiated into calyx and corolla (corolla sometimes absent)
Dichlamydeous flowers	Monochlamydeous and achlamydeous flowers
Choripetaly	Sympetaly (and apetaly except perhaps in Amentiferae)
Pentacyclic flower	Tetracyclic flower
Bisexuality	Unisexuality (except perhaps in Amentiferae)
Monoecism	Dioecism

Perigyny and epigyny appear to be of minor importance in the delimitation of major groups; they may represent simultaneous parallel development in several widely sundered groups.

Sympetaly occurs frequently among the Archichlamydeae, in such relatively primitive families as the Crassulaceae, and all along the line to the Cucurbitaceae; except when associated with other advanced characters (e.g. oligostemony), it has less significance than is sometimes assigned to it, as indicated by the writer in 1935.

A system intended to demonstrate progressive development should be based on those characters considered by its authors to be the least advanced, the sequence proceeding with those considered most advanced. Applied consistently, this principle simplifies the 'natural' classification of families.

It seems unlikely that existing orders and families of Spermaphyta have been derived directly from other living members of these two categories; it is reasonable to suppose that in the long period of time which has been required for existing orders and families to reach their present stage of development, the parental types have disappeared, wiped out by the changes (climatic, tectonic, or catastrophic) which have occurred since the earliest angiosperms appeared. In view of these probabilities it is doubtful whether much is to be gained by assuming the descent of particular groups from other living groups. Perhaps more is to be learned from a study of the directions and degrees of differentiation from a primitive type of flower.

Much of the difficulty of devising any 'natural' system is due to parallelism in the development of groups of different age; progressive development has taken place in some members of a group while other members of the same group have remained relatively stable; development on similar lines may have taken place simultaneously in several groups. A character which may be recognized as relatively 'primitive' (because constantly associated with species or groups known to be relatively unspecialized) may persist in one species, genus or family of a group, when other members of that group have lost that particular character; to that extent the latter have become more 'advanced', though still recognizable as members of the group by the persistence of other relatively primitive characters.

To isolate such advanced members of primitive groups from their obvious associates and to place them together in groups composed only of advanced elements would produce an 'artificial' arrangement; it is customary, therefore, to retain them in the primitive group to which they obviously belong in spite of certain advanced features. In some cases the majority of the component members of a group show relative advance; but the presence in that group of members—or even of only a single member—which retains a primitive character, suffices to indicate the relatively primitive group to which it belongs. A group in which primitive characters are entirely lacking (e.g. tetracyclic oligostemonous Sympetalae) falls into its right place at the advanced end of the linear sequence. In the following arrangement of the higher groups of dicotyledonous orders, an attempt has been made to apply the principles outlined above.

The following outline does not make any provision for exceptions; its purpose is that of a conspectus, to give a general view of the orders of Dicotyledons classified according to their apparent affinities. An effort has been made to arrange the groups on a logical basis by giving precedence to those characters which appear to be the most primitive. At the same time it has been sought to avoid such extreme artificiality in the classification as would ensue if, for example, all cases of parietal placentation were brought into one group.

The important part played by reduction and simplification of structure in the evolution of the modern flower is recognized. Progressive development (as in the direction of adaptations to specialized types of insect pollination) is also taken into account. These two lines of development—often occurring simultaneously in the same group—while forming the basis of classification, must also be recognized as occurring within the individual groups if these are not to become purely artificial. Thus although polystemony is recognized as more primitive than oligostemony, the subsection Polystemonae contains some members in which the androecium has been reduced to a definite number of stamens, e.g. in Capparidaceae.

CONSPECTUS OF THE GROUPS OF DICOTYLEDONS

In the following conspectus the term *family* applies to a collection of genera which are more like each other than they are to any other genera; it is used in the same sense in which Rendle (1925) and Hutchinson (1926) use it, but *not* in the sense given to it by Warming and Potter (1920).

The term *order* is applied, in the same way, to include a group of families in the sense in which both Rendle and Hutchinson use it, but not as Bentham and Hooker (1862–83) or Warming and Potter (1920) use it.

The term *cohort* has been retained for a group of allied orders more or less of the same size as the cohorts of Bentham and Hooker and of Hooker (1873).

A. *The Higher Groups and Cohorts*

Subclass I. Amentiferae (3 Cohorts: 1. Querciflorae; 2. Saliciflorae; 3. Casuariniflorae)
Subclass II. Polystemonae
  Division I. Apocarpicae
    Subdivision I. Polycyclicae (3 Cohorts: 4. Magnoliiflorae; 5. Nymphaeiflorae; 6. Annoniflorae)
    Subdivision II. Oligocyclicae (2 Cohorts: 7. Ranunculiflorae; 8. Rosiflorae)
  Division II. Syncarpicae
    Subdivision I. Axiles (4 Cohorts: 9. Diospyriflorae; 10. Theiflorae; 11. Myrtiflorae; 12. Malviflorae)
    Subdivision II. Parietales (3 Cohorts: 13. Cistiflorae; 14. Rhoeadiflorae; 15. Cucurbitiflorae)
Subclass III. Oligostemonae
  Division I. Pentacyclicae (3 Cohorts: 16. Sapindiflorae; 17. Geraniiflorae; 18. Caryophylliflorae)
  Division II. Tetracyclicae
    Subdivision I. Choripetalae (3 Cohorts: 19. Celastriflorae; 20. Rhamniflorae; 21. Umbelliflorae)
    Subdivision II. Sympetalae
      Section I. Actinomorphicae (5 Cohorts: 22. Apocyniflorae; ('Contortae'); 23. Rubiiflorae; 24. Convolvuliflorae ('Tubiflorae'); 25. Campanuliflorae; 26. Asteriflorae)
      Section II. Zygomorphicae (2 Cohorts: 27. Scrophulariiflorae ('Personales', 'Personatae', *pro parte*); 28. Lamiiflorae)

The progressive development of these major groups and cohorts is indicated in the Table on p. 434.

B. *Principal Orders Included in the Twenty-eight Cohorts*

Cohort
No.
1. Querciflorae: Garryales, Leitneriales, Juglandales, Myricales, Fagales (Balanopsidales?)
2. Saliciflorae: Salicales
3. Casuariniflorae: Casuarinales

TABLE

Classification of Cohorts of Dicotyledons

	Subclass I. Amentiferae.	Subclass II. Polystemonae.				Subclass III. Oligostemonae.			
		Division I. Apocarpicae.		Division II. Syncarpicae.		Division I. 5-Cyclicae.	Division II. 4-Cyclicae.		
		Sub-div. I. Polycyclicae.	Sub-div. II. Oligo-cyclicae.	Sub-div. I. Axiles.	Sub-div. II. Parietales.		Sub-div. I. Choripetalae.	Sub-div. II. Sympetalae.	
Cohort No.								Sec. I. Actino-morphicae.	Sec. II. Zygo-morphicae.
1. Querciflorae	×								
2. Saliciflorae	×								
3. Casuariniflorae	×								
4. Magnoliiflorae		×							
5. Nymphaeiflorae		×							
6. Annoniflorae.		×							
7. Ranunculiflorae			×						
8. Rosiflorae			×						
9. Diospyriflorae				×					
10. Theiflorae				×					
11. Myrtiflorae				×					
12. Malviflorae				×					
13. Cistiflorae					×				
14. Rhoeadiflorae					×				
15. Cucurbitiflorae					×				
16. Sapindiflorae ('Pinnatae') . .						×			
17. Geraniiflorae						×			
18. Caryophylliflorae						×			
19. Celastriflorae							×		
20. Rhamniflorae							×		
21. Umbelliflorae							×		
22. Apocyniflorae								×	
23. Rubiiflorae								×	
24. Convolvuliflorae								×	
25. Campanuliflorae								×	
26. Asteriflorae								×	
27. Scrophulariiflorae									×
28. Lamiiflorae									×

Cohort
No.

4. Magnoliiflorae: Magnoliales, Calycanthales
5. Nymphaeiflorae: Nymphaeales, Cactales, Ficoidales *sensu stricto*
6. Annoniflorae: Annonales, Berberidales, Laurales
7. Ranunculiflorae: Dilleniales, Ranales (Piperales?)
8. Rosiflorae: Rosales (+Connaraceae), Cunoniales, (Saxifragales?), Leguminosae
9. Diospyriflorae: Ebenales
10. Theiflorae: Theales (Ericales?)
11. Myrtiflorae: Hypericales, Lythrales, Myrtales
12. Malviiflorae: Tiliales, Malvales, Urticales, Euphorbiales (Styracales?)
13. Cistiflorae: Bixales (excluding Canellaceae), (Myrsinales?, Escalloniales?, Pittosporales?)
14. Rhoeadiflorae: Rhoeadales, Loasales, Capparidales (+Cruciales), Violales, Tamaricales?
15. Cucurbitiflorae: Caricales, Cucurbitales, Passiflorales, Aristolochiales
16. Sapindiflorae: Rutales, Sapindales, Meliales, Polygalales
17. Geraniiflorae: Geraniales, Malpighiales, (Coriariales?)
18. Caryophylliflorae: Caryophyllales *pro parte*, Chenopodiales, Polygonales, Primulales
19. Celastriflorae: Celastrales, Olacales, Santalales
20. Rhamniflorae: Rhamnales (Proteales?)
21. Umbelliflorae: Umbellales
22. Apocyniflorae: Apocynales, Loganiales, Oleales, Gentianales
23. Rubiiflorae: Rubiales
24. Convolvuliflorae: Convolvulales ('Solanales' *pro parte*), Polemoniales
25. Campanuliflorae: Campanulales
26. Asteriflorae: Asterales ('Aggregatae')
27. Scrophulariiflorae: Scrophulariales ('Personales'; 'Personatae' *pro parte*)
28. Lamiiflorae: Lamiales ('Verbenineae')

Several small orders of more or less doubtful affinity are omitted from this provisional list. Slight rearrangement of certain orders is anticipated.

Detailed discussion of the twenty-eight cohorts and their component orders and families is reserved for a later paper.

SUMMARY

The need for a system of classification in which the series of dicotyledonous orders is collected into groups of orders, has led the writer to propose a revised system of classification. This has been in process of construction during a period of several years.

The researches of Dr. Chalk on the wood anatomy of a large number of genera, scattered through many families ranging over the whole series of the Dicotyledons, have supported much of the rearrangement based on floral morphology.

The method of trial and error has been adopted, with various groupings, to determine which characters indicated the greatest advance, and which of them could be used effectively.

The position of a group has been determined by the presence in it of one or more members having the relatively least advanced character occurring in the group, irrespective of the presence of other members which have lost that character while retaining other features characteristic of the group. On

this basis the sequence shows uniformly progressive development of the twenty-eight cohorts of Dicotyledons.

The class Dicotyledons is divided into subclasses, two of which are determined by polystemony and oligostemony, respectively.

The subclass Polystemonae is subdivided into Apocarpicae and Syncarpicae, on the character of the gynaeceum; the monocarpellary gynaeceum of many Rosales and of the Leguminosae is considered to represent reduction from a polycarpellary type.

The division Apocarpicae is subdivided into the Polycyclicae and Oligocyclicae, on the basis of a multiseriate perianth, as in Magnoliaceae, or a biseriate perianth, as in Ranunculaceae.

The division Syncarpicae is subdivided into the subdivisions Axiles and Parietales, on the placentation.

The subclass Oligostemonae, in which oligostemony is constant, is divided into Pentacyclicae and Tetracyclicae, based on the presence of two or of only one whorl of the androecium. The cohorts of Pentacyclicae are not further grouped.

The Tetracyclicae are subdivided into Choripetalae and Sympetalae on cohesion of the petals. The cohorts of the Choripetalae are not further grouped. The Sympetalae are subdivided on progressive development of zygomorphy in the corolla, into Actinomorphicae and Zygomorphicae; this is recognized as perhaps the least satisfactory grouping of the whole arrangement.

The Amentiferae are treated as a separate subclass, of possibly distinct phylogenetic origin, and the group is placed at the beginning of the whole series on account of its incomformity with other groups, and its apparent age, many of its members being contemporaneous with the Magnoliales and Laurales in Cretaceous times, and showing undifferentiated wood-structure.

It has been found convenient to retain the orders as reduced in size by Hutchinson (6), but to collect them into large groups corresponding more or less to the cohorts of Bentham and Hooker (2), for which the term cohort has been retained.

The above arrangement shows uniformly progressive development of the cohorts based on floral structure.

The writer is indebted to Professor Tansley, Professor Matthews, Professor Troup, Dr. Bancroft, and Mr. A. C. Hoyle, for valuable suggestions and criticism of the first draft of this paper.

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The Problem of Biscutella laevigata L.

II. The Evidence from Meiosis

BY

IRENE MANTON

(Department of Botany, University of Manchester)

With Plates XIX and XX, and six Figures in the Text.

INTRODUCTION

THE work on Biscutella is a study of the recent evolutionary history of a natural wild type as revealed by its cytology. The initial facts have already been published (Manton, 1934) and for details the reader must be referred to that paper. A brief summary of the conclusions will, however, be given since they form the starting-point of the present investigation.

It was shown in the earlier paper that the so-called species *B. laevigata* L. was a composite one containing at least three cytological forms each with a distinct geographical range. A hexaploid ($6n = 54$) was recorded from Spain; the population over most of the Swiss and Austrian Alps was shown to be tetraploid ($4n = 36$), while diploids ($2n = 18$), with the one exception of a very local alpine in the mountains near Vienna (var. *mollis*), were lowland forms, spread discontinuously from Germany to the Balkans, in unglaciated areas to the north and east of the Alps. These facts constitute the problem, and attention has primarily been directed to elucidation of the mutual relationships of the central European forms, both tetraploid and diploid. The problem of the tetraploid is that of its origin and parentage. The problem of the diploids is that of their disrupted area. Both have the added interest of a relationship to glacial phenomena by which a rough estimate of age can be made. On this basis it was suggested that the alpine tetraploid population must be the result of recent immigration, while the lowland diploid populations might be relicts of an older flora. On all these points conclusions have been reached by means of the new data to be presented, while a small amount of work has been done on the Spanish hexaploid and on other species of the genus.

METHODS

The procedure adopted has been, in the first place, the establishment of hybrids between all available forms of the subsection Laevigatae (cf. p. 441 for details of stock material) and, in the second place, the investigation of the

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mutual homologies of the chromosomes by observation of their methods of pairing at meiosis.

The polyploid hybrids raised included triploids of all possible combinations of diploid and tetraploid forms. Some pentaploids were obtained from hexaploid ♀ × tetraploid ♂, and the hexaploid was also fertile with some diploids. An extensive series of inter-diploid crosses was also set up.

The technique of hybridizing was simplified by the presence of strong self-sterility in all the forms examined and by the very early date of flowering, February, under greenhouse conditions. A chromosome count was regarded as sufficient evidence for the correctness of the cross in all cases where the hybrid number was different from that of the parents. In no case was any error found. Greater care against possible contamination was exercised where an error could not be revealed cytologically, i.e. in inter-diploid crosses. All of these were castrated, many of them were bagged, though morphological abnormalities were sometimes induced by this, and finally a control series of crosses were repeated under most stringent conditions of castration and bagging. Very often the correctness of the cross could be demonstrated on morphological criteria, and all the cytological results were in agreement.

The cytological results are represented photographically in the plates, though some photographs, notably those of the complete series of polyploid roots, have appeared elsewhere (Manton, 1935) and are not repeated. Notes on technique will be found in the legends, but in general it may be said that every ordinary method required modification for success. Wherever possible haematoxylin was used both for smears and for sections on account of its permanence, a quality in which it is immensely superior to gentian violet. Details of staining were those advocated by Dame Helen Gwynne Vaughan (1927) with various strengths of alum, an 8 per cent. mordaunt being used for roots and 2 per cent. for mother-cells. For smears the best fixative was 2 BD (La Cour, 1931) diluted with half its volume of water. For sections of buds 2 BE with doubled osmic acid was satisfactory for diploids; for the higher polyploids better results were sometimes obtained with the doubling of both osmic and acetic acids in the 2 BE formula. Pretreatment with Carnoy's fluid was actively detrimental for diploids, but was sometimes advantageous for the higher numbers. 2 BE was used for roots. Sections varied from 6μ–12μ according to the size of cell expected.

For the quantitative work on chromosome pairing very special value was found in a modification of the permanent acetocarmine methods of McClintock and Heitz. Material was fixed in Carnoy overnight and the mother-cells then squashed out on to a slide and boiled violently in a drop of fresh acetocarmine under a coverslip. By this means it is possible, as can be seen from the plate, to flatten and spread the whole contents of a cell so that every chromosome in a metaphase side-view (e.g. Pl. XX, Fig. 24), or even in all the four nuclei of a tetrad (Fig. 26), can be recorded in one focal field. All these photographs are of balsam mounts finished off according to McClintock's method (McClintock, 1929). Attention should perhaps be drawn to the swelling of the chromosomes by the acetocarmine. This can be watched under the microscope and does not appear to involve any other distortion. Where chromosomes are as small as in this plant this artifact may be of great use, but it has involved the choice of a lower magnification for these figures.

The Stock Material

As in the first paper, all the parental stocks were wild plants of known origin. The identity in each case was, as before, checked by the author of the most recent systematic monograph, Frau Machatschki-Laurich, or the specimens were from type localities which admitted of no confusion. In most cases a herbarium record was kept and the collection is now housed in Manchester Museum.

All the tetraploids and the hexaploid were those previously used, the provenance of the first being various localities in the Alps of Austria and north Italy, the second being the Picos de Europa (province of Santander) in Spain. Among the diploids three subspecies of *B. laevigata* have been obtained in addition to those previously examined. They are subsp. *questphalica* from the Süntel in Brunswick, subsp. *tenuifolia* from the Harz Mountains, and subsp. *austriaca* from Vienna. These therefore complete the diploid forms of *B. laevigata* sensu Machatschki-Laurich, since the only subspecies still missing, subsp. *illyrica*, is expected to be tetraploid.

A more significant addition to the diploid material has been the collection of other species of the Laevigatae group from their type localities in the area to the west of the Alps, namely, central and southern France (see map, Text-fig. 1). *B. divionensis* Jord.,[1] *B. mediterranea* Jord.,[2] *B. controversa* Boreau,[3] *B. coronopifolia* L.,[3] *B. Lamottii* Jord.,[3] and *B. arvernensis* Jord.[3] have all been investigated and all have eighteen chromosomes (see Pl. XIX, Figs. 13 and 14).

Finally, *B. tomentosa* Lag[4] has been received from the rock of Gibraltar. It also has eighteen chromosomes (Fig. 19). This plant is of special interest, for it comes from practically the southernmost limit of the Laevigatae group, only two species of which are recorded at all from north Africa (i.e. *B. frutescens* and *B. sempervirens*). In spite, therefore, of the regrettable absence of other material from Spain, it is highly probable that the Laevigatae stock in that country, as in others, is a homogeneous one. In the rich assemblage of species on the Spanish east coast it may be expected that the number eighteen will recur, and the same expectation holds for the little species *B. brevicaulis* Jord. from the French Alps, which is also still missing.

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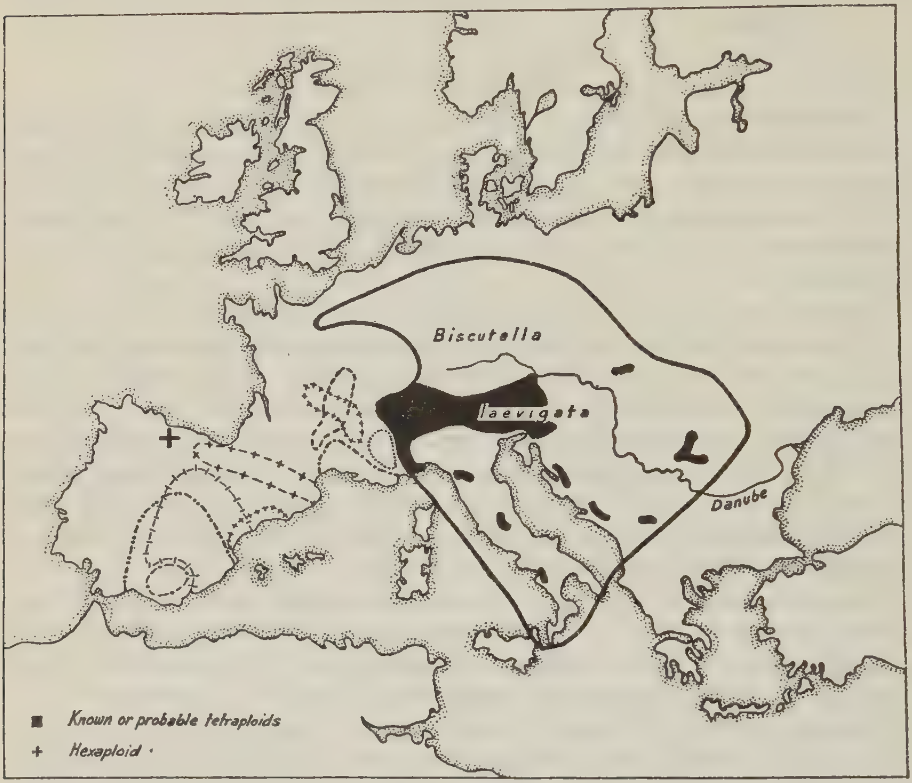
[1] From near Dijon. [2] From Montpelier. [3] From the Auvergne.

[4] This species is regarded by Machatschki-Laurich as included in *B. montana* Cav. which she quotes only from farther north on the mainland. This discrepancy has made it seem preferable to retain Lagasca's name in the present instance since Gibraltar is the type locality for the plant described by him.

MEIOSIS IN THE EVEN POLYPLOIDS

It was shown in the first paper that in nature the tetraploid forms of *B. laevigata* do not breed quite true but throw individuals with one chromosome too few or too many in about 20 per cent. of seedlings. The same is true to an even greater extent in the hexaploids. Only two regular specimens with fifty-four chromosomes were found among seven wild plants, other numbers counted being three plants with fifty-five, one plant with fifty-six, and one plant with fifty-three.



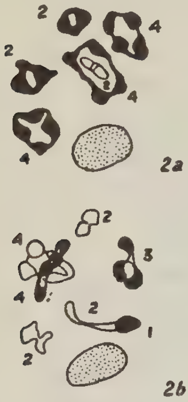
TEXT-FIG. 1. Distribution of species of Biscutella in the subsection Laevigatae after Machatschki-Laurich, 1926 (p. 114). Reproduced from Text-fig. 2 of Manton, 1934, with data derived from both papers inserted. Symbols referring to species quoted in the text are + + + + *B. coronopifolia* L., ... *B. brevicaulis* Jord., - - - - *B. controversa* Bor. —·—·— *B. mediterranea* Jord., —··—··— *B. montana* Cav., ——— '*B. laevigata*' L. (sensu Mach-Laur). Note that the range of the tetraploid is partly assessed on morphological grounds (cf. Manton 1934, text-fig. 5) but the diploid sites are actual sources of cytological material.

This instability at once suggests the presence of multivalent groups at meiosis, and in both polyploids these can be clearly shown to occur. Pl. XIX, Fig. 1, is from a section of a tetraploid plant with the morphology of subsp. *longifolia*. It shows a portion of a side-view of a pollen mother-cell in the irst division, with a pair and a quadrivalent in position on the spindle and a trivalent and a single chromosome slightly out of focus to the left. The appearance of these groups at diakinesis is shown in Pl. XIX, Figs. 2–4, which are three focal levels of a cell from another plant. Figs. 7 and 8 of the diploid subspecies *alsatica* are added for comparison. The pair of chromosomes of Fig. 7 contrasts very clearly with the quadrivalent ring of Fig. 4 (which also contains two pairs). Fig. 3 has two quadrivalent rings in focus at top and bottom, while Fig. 2 shows a trivalent and a single (on the right). Similar qualitative demonstration of multivalent groups in the hexaploid is given in Figs. 9 and 10. These are two focal levels of the same nucleus in sectioned material, Fig. 9 showing a ring of six, and Fig. 10 a quadrivalent. Pl. XX, Fig. 40, is a portion of an acetocarmine squash containing a zigzag hexavalent and a ring of four.

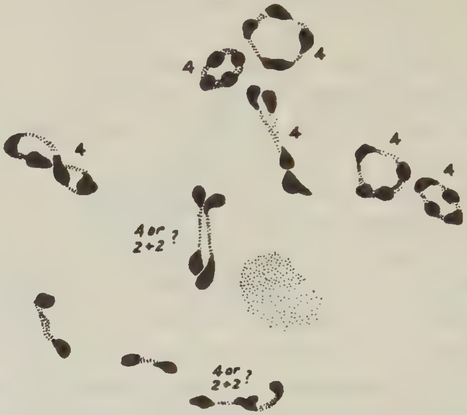
The effect of this type of pairing on the numerical distribution of chromosomes in the tetraploid is given in three photographs. Fig. 28 is an anaphase of the first division of a pollen mother-cell in acetocarmine showing eighteen chromosomes going to each pole. Pl. XIX, Fig. 12, on the other hand, shows a cell in the second division with seventeen chromosomes in the upper plate and nineteen in the lower. Two such cells were found among fifteen counted, a number commensurate with the frequency of aberrant individuals. Fig. 15, finally, is from the root of a triploid hybrid with twenty-eight instead of twenty-seven chromosomes. The female parent of this plant was a normal tetraploid with thirty-six chromosomes. The extra chromosome of the hybrid thus denotes the production of unbalanced female gametes in the same manner as in the male. Similar demonstration could have been obtained for the hexaploid, but only Fig. 11 need be quoted. This shows $3n+1$, i.e. twenty-eight chromosomes at the second division in a pollen mother-cell. This figure is not quite comparable with that of the tetraploid (Fig. 12) because the plant was itself an irregular hexaploid with $6n+1$, i.e. fifty-five chromosomes. It is, however, sufficient to show that the observed instability of both polyploids in nature is of the same order.

Quantitative data for the proportion of multivalent groups in completely analysed nuclei have been obtained for tetraploids but not for the hexaploid. The analysis of the photographed nucleus already referred to (Figs. 2–4) may be made from Text-fig. 2. It contained five quadrivalents, six pairs, a trivalent, and a single. Text-fig. 3 gives the analysis of another cell from the acetocarmine squash photographed in Pl. XX, Fig. 27. This nucleus is of particular interest, for the plant it represents is the same individual, with the morphology of subspecies *lucida*, whose somatic chromosomes were photographed in the first paper. It is also of interest for the high number of quadrivalents displayed. These at first sight appear to be eight, with two discrete pairs, and this is quite likely to be the correct interpretation. Two of the multivalent groups are, however, marked with queries, for it is not quite certain from their shapes whether they are true quadrivalents or accidental associations of pairs forced into contact by the action of squashing. This nucleus is thus not absolute proof of more than six quadrivalents.

The numbers of multivalent groups in ten cells of this individual are summarized in Table I. Multivalents include both quadrivalents and trivalents, though the latter are much in the minority, being rarely more than one per cell (as in Text-fig. 2) and never more than two. Unpaired chromosomes only occur in relation to such trivalents. While the number of cells studied is too few for statistical treatment, it is evident that the commonest number of multivalents is that which involves 50 per cent. of the chromosomes, i.e. four or five multivalents out of a maximum possible of nine. The range of variation is considerable, though no cell without any multivalents has yet been seen. Examples of cells with only one multivalent are also absent from the table, but one such was found in sectioned material; the data from sections are, however, not incorporated as being less accurate in the higher values. It is, however, precisely the higher values which are of the greatest interest for the evidence they give on chromosome identity, since the power to form a quadrivalent, even rarely, must denote a measure of homology between all



TEXT-FIG. 2.



TEXT-FIG. 3.

TEXT-FIG. 2*a* and *b*. Diagrammatic analysis of the tetraploid nucleus photographed in Pl. XIX, Figs. 2–4, showing pairing of chromosomes at diakinesis in tetraploid subsp. *longifolia*. × 2,000.

TEXT-FIG. 3. Analysis of the cell photographed in Pl. XX, Fig. 27, showing chromosome pairing at diakinesis in tetraploid subsp. *lucida*. × 2,000.

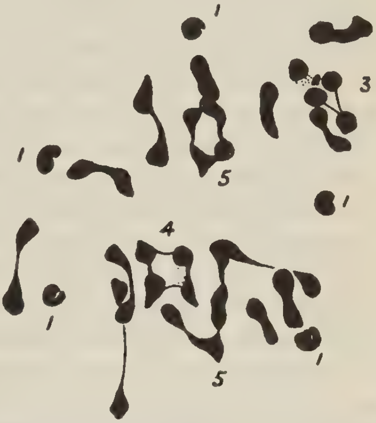
TABLE I

Numbers of Multivalent Groups in Ten Cells of the Same Plant of Subsp. lucida *previously dealt with. Data from Acetocarmine Smears*

Number of multivalents:	0	1	2	3	4	5	6	7	8	9
Number of cells:	—	—	1	1	3	3	1	—	1?	—

the four chromosomes composing it. Of these higher values the two cases quoted in the table are representative of the available evidence. It has not so far been possible to demonstrate with certainty numbers as high as 7, 8, or 9. The reason is in part technical, as has been explained in connexion with the doubtful case of eight analysed above, and negative evidence is thus no proof of their absence. The positive evidence is, however, quite conclusive as far as it goes. There is no doubt at all that both subsp. *lucida* and subsp. *longifolia* do, not infrequently, form six quadrivalents; both subspecies must therefore possess at least six of their chromosomes in quadruplicate. This proportion is sufficiently high to be strongly suggestive of autopolyploidy.

MEIOSIS IN THE UNEVEN POLYPLOIDS



TEXT-FIG. 4. Analysis of pentaploid nucleus photographed in Pl. XX, Fig. 30, showing pairing at the first meiotic division. For clearness only singles and multivalents are labelled; pairs are not numbered. × 3,000.

Of the uneven polyploids the pentaploids may be passed over briefly, since it has not been possible to study them very closely. Five plants were raised from three crosses with one hexaploid individual from the Spanish stock. In one cross, which gave three of the plants, the hexaploid was used as male parent; in the other two crosses, each of which produced one viable seed, the hexaploid acted as female. The three tetraploids involved were sister plants from family '33' quoted in full in the first paper. They belonged morphologically to subsp. *longifolia* and came from the Karer Pass (north Italy). The chromosome number of the hexaploid was afterwards found to have been 53, i.e. $6n-1$. The chromosome numbers of the hybrids were: three plants with 44 (i.e. $5n-1$), one plant with 45, and one plant with 46. Meiosis was chiefly studied in one of the $5n-1$ plants and some photographs are included in the plates. Configurations in all valencies from one to five chromosomes can be found at diakinesis and the first metaphase. Pl. XIX, Figs. 5 and 6, show a quadrivalent and a pentavalent at diakinesis in sectioned material, and Pl. XX, Fig. 30, is of an acetocarmine squash. The analysis of this can be made from Text-fig. 4, which shows it to contain two pentavalents, one quadrivalent, probably one trivalent, eleven pairs, and five singles. The distribution of chromosomes is irregular, as in all uneven polyploids, and Fig. 31 shows a cell with twenty-five chromosomes going to one pole and ten to the other, while four unpaired chromosomes have lagged on the spindle and are dividing. These prematurely split chromosomes will not divide again but are distributed at random at the next division. A tetrad from the same plant is shown in Pl. XX, Figs. 32*a* and *b*, in which the four nuclei have 22, 23, 22, 21 chromosomes in order from left to right. Judged by the setting of seed under free pollination, the pentaploids appear to be more fertile than the triploids, but it has not been possible to study their breeding in detail. The only evolutionary deductions which can be made from these somewhat scanty facts are that the relationship between the polyploids of Spain and of central Europe is not a distant one, and that at least three out of the nine haploid chromosomes are common to both.

As opposed to the pentaploids, very special attention has been given to the triploids, since they are the key to the whole problem. The first point of interest was to ascertain the degree of inter-fertility between the tetraploid and all available diploids. Starting with subsp. *alsatica*, crosses were made in both directions with all named forms of tetraploids, i.e. subspp. *longifolia* and *lucida* and vars. *obcordata*, *tyrolensis*, and *angustifolia*. In spite of the limited number of seeds in the ovary (only two), this crossing proved to be unexpectedly easy. Certain individuals are mutually sterile, an expected consequence of self-sterility, but apart from this, inter-fertility is so great that under conditions of free pollination in Professor Weiss's garden triploids were spontaneously produced. No difference could be detected in the behaviour of the different tetraploid strains, which were thereafter treated as equivalent.

Of the other diploid forms of *B. laevigata* (in the sense of Machatschki-Laurich) all but two were successfully incorporated as triploids, the missing pair being subsp. *gracilis*, which died out early, and subsp. *guestphalica*, which has only recently been acquired. The tetraploid is thus fertile with subspp. *austriaca*, *tenuifolia*, *subaphylla*, *kerneri*, and var. *mollis* in addition to subsp. *alsatica*. The viability of all these triploids is good except for the one incorporating var. *mollis*. This variety is a diminutive alpine which flowers very sparingly in cultivation and produces very small seedlings. There is always a risk of loss of seedlings by damping off, and of this combination only one plant has survived, but it is still too young to have flowered.

In addition to these diploids of the same 'species', tetraploid *B. laevigata* has proved to be fertile with the whole range of other so-called species from central and southern France, and seed has been set with *B. tomentosa* from Gibraltar, though these have not yet germinated. The French species comprise *B. mediterranea*, *B. divionensis*, *B. coronopifolia*, *B. controversa*, *B. arvernensis*, and *B. Lamottii*. Their triploids are as viable as the others, though *B. Lamottii* introduces the same kind of difficulty as does var. *mollis*. This species is not a dwarf but is very slender in all its parts and has small flowers. Seedlings incorporating it are diminutive and liable to damp off, but since it flowers profusely pollinations can be multiplied and several triploids with it have reached maturity. This extended inter-fertility of the tetraploid was unexpected, but it is of considerable importance in understanding the phylogeny of the group.

Meiosis in eight of these triploid combinations has been successfully studied. The general features are, as in the pentaploid, that all paired chromosomes segregate at the first division while singles lag and divide. The segregation is often unequal owing to the formation of trivalents (Pl. XX, Fig. 24), and Fig. 25 shows twelve chromosomes at one pole and nine at the other, with six laggards in the act of splitting. Fig. 26 is of a tetrad in which the four nuclei have 12, 13, 15, 14 chromosomes, in the order from left to right, the fact that no two are alike being due to the further random segregation of the singles at the second division. These photographs provide very clear demonstration of the general phenomenon of triploid meiosis well known from other plants, but they are not the main line of inquiry. This is primarily to determine the extent of trivalent pairing in the various types of hybrid.

TABLE II

Frequency of Trivalents among Seventy-three fully analysed Cells from Thirteen Triploid Plants involving Eight Different Forms of Diploids. One Photograph from each kind of Triploid is given in Pl. XX

Name of diploid component.	Number of trivalents:										Total no. of cells.
	0	1	2	3	4	5	6	7	8	9	
subsp. *alsatica* (3 plants)	—	—	1	2	1	6	2	3	—	—	15
subsp. *kerneri* (3 plants)	—	—	—	—	1	—	2	—	—	—	3
subsp. *tenuifolia* (1 plant)	—	1	2	3	6	—	1	1	—	—	14
subsp. *austriaca* (1 plant)	—	—	—	—	—	1	1	4	—	—	6
B. coronopifolia (1 plant)	—	—	—	2	3	6	1	2	1	—	15
B. controversa (2 plants)	—	—	—	—	—	2	2	—	1	—	5
B. Lamottii (1 plant)	—	—	—	2	—	—	—	1	1	—	4
B. mediterranea (1 plant)	—	1	1	3	1	3	1	1	—	—	11

The evidence on this line is collected in Table II and in the plate. Pl. XX contains a photograph of one completely analysable cell from each kind of triploid, the series (Figs. 24 and 33–9) representing triploids with *B. coronopifolia*, *B. controversa*, *B. mediterranea*, *B. Lamottii*, and *B. laevigata* subspp. *alsatica*, *austriaca*, *kerneri*, and *tenuifolia*. All of these photographs are sufficiently clear to be analysed at sight, with the aid of the legends, and explanatory diagrams are superfluous. Fig. 24, from the triploid with *B. coronopifolia*, shows five trivalents; Figs. 33 and 38, representing *B. mediterranea* and *B. controversa*, show six, while all the rest show seven, with the possible exception of Fig. 34, which may really have eight. The background to these figures is provided by the table. The photographed cells have been selected both for flatness of field and for high numbers of multivalents. Actually the variation in numbers is very great, as in the tetraploid, though the extremes (no trivalents and nine trivalents) have not been seen. As in the tetraploid, it is not possible, from the nature of the material, to work on a scale suitable for statistical treatment. Nevertheless, the table in conjunction with the plate provides a body of evidence of a very definite kind. It is clear that all the diploids used are capable of forming trivalents in considerable numbers with the tetraploid, six having been seen in each case and, in some, seven or eight. The status of the diploids, whether independent species or subspecies of *B. laevigata* itself, is apparently immaterial.

THE DIPLOID HYBRIDS

Evidence confirmatory of all the foregoing results has been obtained from the behaviour of the diploids with each other. Of the eleven types incorporated in the triploids, *B. mediterranea* died out before it could be used, but all the rest have been crossed with each other in as many combinations as was practicable. Twenty-three of these hybrids have been examined cytologically, their constitutions being as follows:

B. coronopifolia ♀ × *B. Lamottii*, *B. arvernensis*, *B. controversa*, and *B. laevigata* subspp. *tenuifolia*, *alsatica*, *kerneri*, *austriaca*, and var. *mollis*.
B. controversa ♀ × *B. Lamottii*, *B. arvernensis*, and *B. laevigata* subspp. *kerneri* and *tenuifolia*.
B. arvernensis ♀ × *B. controversa*, *B. coronopifolia*, and *B. laevigata* subsp. *tenuifolia*.
B. Lamottii ♀ × *B. controversa* and *B. laevigata* subspp. *alsatica* and *subaphylla*.
B. laevigata subsp. *alsatica* ♀ × subsp. *kerneri*.
B. laevigata subsp. *kerneri* ♀ × subsp. *alsatica*.
B. laevigata subsp. *austriaca* ♀ × subspp. *tenuifolia* and *alsatica*.
B. laevigata subsp. *tenuifolia* ♀ × *B. Lamottii*.

All the cytological results are uniform, there is no trace of failure of pairing anywhere, and as samples of the evidence the three photographs, Pl. XX, Figs. 21–23, will suffice. These are all inter-specific hybrids between *B. laevigata* diploids and species from France.

PHYLETIC CONCLUSIONS REGARDING THE POLYPLOIDS

All this evidence admits of only one interpretation. It is that all forms of Biscutella in France and central Europe (probably also in Spain) belong to a closely related stock. Polyploidy, where it has occurred, is indistinguishable cytologically from autopolyploidy. At the same time it must be recognized that the diploid stock is undoubtedly splitting up into distinct morphological and ecological units to which separate names have, quite rightly, been given. Genic mutations must be assumed to underlie this, since it has not been possible to detect any gross cytological changes other than polyploidy. Genic mutations have also been suggested by the evidence of segregation of characters in the tetraploid populations mentioned in the first paper (loc. cit., p. 54). Heterozygosity in the parentage of the tetraploid is therefore not excluded by the cytological evidence of autopolyploidy.

The origin of the tetraploid can therefore only be tracked down more precisely on genetical criteria. Fortunately some evidence of this nature is obtainable from the morphology of the plants. Attention was drawn in the first paper to the tendency to proliferate root-buds shown by the tetraploids in cultivation, and to an even greater extent by the hexaploids. This character has been found in nature in the diploid species *B. arvernensis* and *B. Lamottii*, both strictly localized forms confined to central France. The same character is strongly suggested by Machatschki-Laurich's description of *B. brevicaulis* from the French Alps, a form not seen by me. In every other diploid that I have examined branching appears to be exclusively from the stem. It is therefore highly probable that the tetraploid has not arisen directly from any of the diploids of Germany or Austria, but that its affinities are closer with the diploids of France and that it entered the Alps from the west or south-west.

Three further facts are relevant to the origin of the tetraploid. The frequency of occurrence of chimaeral tissue in roots has been referred to in another connexion (Manton, 1935); among the diploids, chimaeras have been found in *B. divionensis*, *B. tomentosa*, and '*B. laevigata*' subspp. *tenuifolia* and *austriaca*. An incipient chimaera of the type recently photographed in *Iberis semperflorens* (Manton, 1935) is shown in Pl. XIX, Fig. 14, from *B. divionensis*. Chimaeral tissue appears to be most frequent in adult plants which have been dug up and sent by post and may be partly the result of shock, but the possibility that such a chimaera might result in a tetraploid plant is obvious, especially where proliferation of root-buds can occur. A second mode of origin, from unreduced gametes, is, however, possible, though for the tetraploid less likely. Such gametes have been seen very sparingly in all the polyploid grades except diploids, and one example, in a triploid, is given in Fig. 16.

The last relevant fact concerns the hexaploid, though it must be used with caution, as will appear. The hexaploid is distinguishable from all the other stocks in use by the gigas nature of its flowers and fruits and by its very long, rigid, leafy, and not very erect, flowering axes. These in cultivation may extend to nearly 2 ft. and bear a succession of leafy bracts up to 2 in. long. When crossed to the tetraploid, pentaploids result as already described. When crossed to diploids. seed has been set with subspecies *kerneri*, *alsatica*, and *tenuifolia* and with *B. coronopifolia*. Two specimens of the last cross have been successfully germinated and raised to maturity. They are tetraploid in number (Pl. XX, Fig. 29) and form quadrivalents at meiosis. One has an extra chromosome, and the occasional presence of a pentavalent even in the other indicates that both are unbalanced. This is probably an incidental consequence of an extra chromosome known to have been possessed by the hexaploid parent. Genetical analysis of these hybrids has not been attempted, hence the word of caution above, but the general impression they give is of a strong morphological resemblance to their hexaploid rather than to their diploid parent or to the true alpine tetraploids. This suggests that the hexaploid is probably genetically different from the alpine tetraploids, in spite of its cytological affinity.

The Question of Date

The question of date remains to be considered. This is admittedly a matter more of theoretical conjecture than provable fact, but on the whole it would appear that the evidence put forward in the first paper is not invalidated by the new material. The probability is still clear that the tetraploid has spread over the Alps post-glacially and may still be expanding its range, while many of the diploids to the north of the Alps are very local and appear to be relicts of an earlier immigration. Since they are 'xerothermal' relicts, it must be supposed that they arrived under conditions of greater warmth than those prevailing at present, while their peculiar relation to unglaciated areas suggested that this warm period was not a post-glacial one.

The sum of the evidence now available points more precisely to an inter-glacial rather than to a pre-glacial date for this immigration. The key to the diploid problem in central Europe would appear to be 'var. *mollis*'. This little alpine, though classed by Machatschki-Laurich as merely a variety of subsp. *austriaca*, is in fact the most distinct morphologically of all the diploids used. It becomes laxer in all its parts under greenhouse conditions but retains very markedly its diminutive character. Ecologically it is strictly confined to the truly alpine habitat, above the tree line and in association with such species as *Campanula alpina*, *Ranunculus alpestre*, *Androsace* spp. Though locally abundant, as on the Wiener Schneeberg, it appears to be confined to relatively few peaks in the eastern Alps and possibly Roumania. The present spacial separation from subsp. *austriaca*, which is a lowland form, makes it difficult to believe that the one can have arisen from the other under existing conditions. It seems far more likely that 'var. *mollis*' came into contact with its alpine associates not after, but during, the glacial phenomena and that it reached its present stations along with the rest of the alpine community. On the other hand, a very great antiquity is precluded by its relatively small geographical range. It must therefore be concluded that the most probable date for the advent of 'var. *mollis*' is the last glaciation; the most probable date for the advent of the diploid stock into Austria is the last inter-glacial period.

One other point which should perhaps be mentioned is the opinion of Braun-Blanquet (1923, p. 331) that the endemic forms found in the Auvergne, namely, *B. arvernensis* and *B. Lamottii*, are incipient species of recent origin. This may well be so, for the dating of the central European relicts does not directly affect the situation in France. Unrestricted access from the unglaciated

Mediterranean may have led to waves of migration entering France at different times. It is not impossible indeed that *B. coronopifolia* and its allies may represent just such a different wave from that which introduced *B. laevigata*. This, however, is pure speculation which could only be settled by genetical analysis on the spot.

RECOMMENDATIONS ON NOMENCLATURE

It is proposed to pass very briefly over questions of taxonomic status largely because of their extreme difficulty. It is clearly impossible to reach uniformity in the conception of what shall constitute a species[1] when even precisely defined terms such as auto- and allo-polyploid tend to lose their clarity when applied to natural cases, as in the present instance. Nevertheless, although cytological criteria alone are insufficient basis for taxonomy, it often happens that both ecological and morphological differences are associated with differences of chromosome number. As has been pointed out by Muntzing in a very valuable recent summary (1936), these differences may also be associated with sterility barriers, even in so-called auto-polyploids. All these features are shown by the polyploid Biscutellas and, in addition, differences of date and place now separate them from the diploids with which, on the present taxonomic system, they are associated. There is evidence, moreover, that the diploids which now go under the same specific name as the polyploids are not those with which they have the closest genetic affinity. On any definition of species, the inclusion of all these forms under the one name *B. laevigata* is thus actively misleading. Some curtailment of the limits of this 'species' should therefore be effected in any taxonomic revision of the genus and, in the hope of facilitating such revision, the following recommendations are made to indicate the kind of change which would be consistent with the new knowledge:

I. The constancy and distinctness of var. *mollis* Schur. suggests that it has as good a claim to specific status as has *B. Lamottii* Jord. The restoration of this variety to specific rank is therefore recommended, and its name should then be *B. minor* Jord.[2]

II. It is recommended that the tetraploid forms designated in Machatschki-

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[1] The difficulty of standardizing the species concept has recently led a number of experimental taxonomists into advocating the use of an entirely separate system of units to express their results. The system most in favour seems to be the ecological one of Turresson, and definitions of 'coenospecies', 'ecospecies', and 'ecotype' are given by Gregor, Muntzing, &c. Though easy to use and no doubt adequate for their original purpose, these definitions when applied to Biscutella lead to an artificial result. Of the forms here studied, the whole of the subsection Laevigatae would constitute a coenospecies, the polyploids and they alone would be ecospecies, while all the distinguishable diploid forms would be ecotypes. The stress which is thus laid on the polyploids is due to the manner in which sterility is used as a diagnostic character, but sterility, though inherent in the polyploid state in certain combinations, is, in them, of only incidental significance. The polyploids are in other ways no more distinct, self-contained, or specialized than many of the known diploids. The indiscriminate use of the ecological system as defined by Gregor is thus in my opinion misleading.

[2] I am indebted to Dr. Turrill and Mr. Gilmour of Kew for verification of questions of priority in nomenclature.

Laurich's monograph as *B. laevigata* subspp. *lucida*, *longifolia*, *illyrica* (?), and the varieties within these subspecies, should collectively be given specific rank. This species should retain the name *B. laevigata* L. emend., since examination of the Linnean Herbarium has shown that Linneaus probably had only a tetraploid specimen of the *longifolia* type before him when he proposed the name.[1]

III. For the remaining diploids designated by Machatschki-Laurich as *B. laevigata* subspp. *alsatica*, *austriaca*, *kerneri*, *gracilis*, *subaphylla*, *tenuifolia*, and *guestphalica*, the earliest available specific name appears to be *B. austriaca* Jord. emend.[1] In recommending its adoption it is, however, pointed out that the cytological evidence here provides no features of classificatory value and the question of further reassessment of specific boundaries among the diploid species and subspecies is still open.

IV. Separation from *B. laevigata* of the high polyploids of northern Spain is also probably desirable, though these forms are still incompletely known.

Evolution in the Genus as a Whole

Throughout this investigation, which has been the principal work of five years, every effort has been made to collate the results for the group under intensive study with some comparative data for other members of the genus. The background thus provided has been so instructive that it is profitable to present it briefly before summing up.

The genus Biscutella, according to the scheme retained by Machatschki-Laurich, is divided taxonomically into two main sections known respectively as Iondraba and Thlaspidium. The former has *B. auriculata* L. as its type, an annual plant with pouched sepals and sixteen chromosomes (Manton, 1932). The distribution of this section, according to Malinowski (1910), is in the western Mediterranean countries from Dalmatia to north Africa and also in the Balearic Islands, the Canaries, and Teneriffe. The presence of *B. auriculata* on these islands, where it is the sole representative of the genus, has been quoted as evidence for the considerable antiquity of this section, which is thus regarded as the oldest of the existing types. It is not possible to postulate a date for it without more knowledge, but it is a well-known fact that the present flora of the Canaries is closely akin to the flora of France (Cantal) in the Pliocene and early interglacials (down to the Mindel-Riss, but not later). It is thus not impossible that the section Iondraba may date back to those epochs.

The section Thlaspidium contains, on the one hand, the group of perennials with eighteen chromosomes constituting the subsection Laevigatae and on the other a group of annuals known as the Lyratae. The Lyratae differ from the Laevigatae in their annual habit, in being self-fertile, and in various morphological details of petals and nectaries besides their lyrate leaves. They are also stated to have more hairy cotyledons, a fact which is probably correlated with rather greater xerothermal requirements, for all are heat lovers and several are semi-desert plants. Both Malinowski and Machatschki-Laurich agree, in regard to the relative ages of the two subsections, that the Lyratae and not the Laevigatae are the older. For this reason it has seemed particularly desirable to reinvestigate the cytology of the Lyratae as far as possible from wild material of known origin, and the attempt has been so far successful in that five of the seven species have been freshly collected, largely by the kindness of correspondents. The sixth species, *B. eriocarpa*, is still only known from Botanic Gardens material (Manton, 1932), but *B. radicata* Cosson alone is missing.

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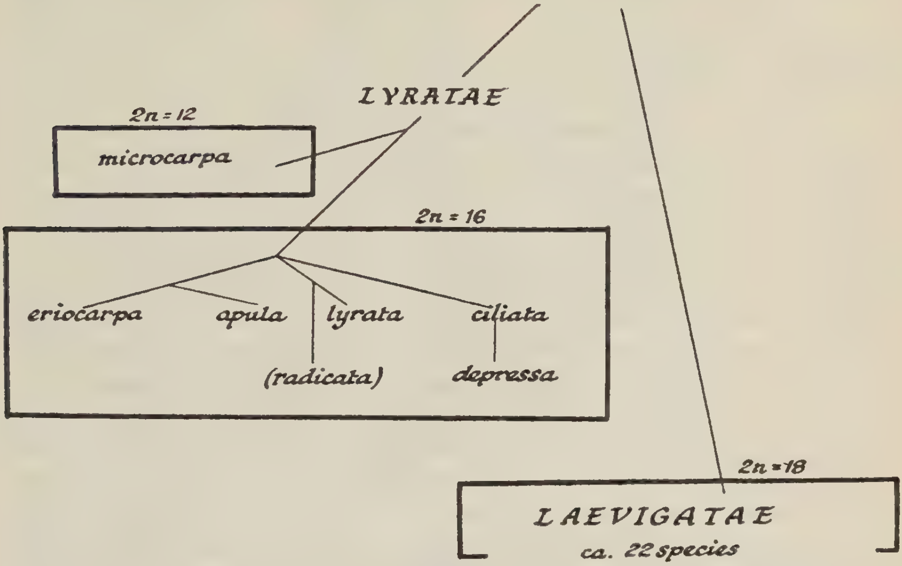
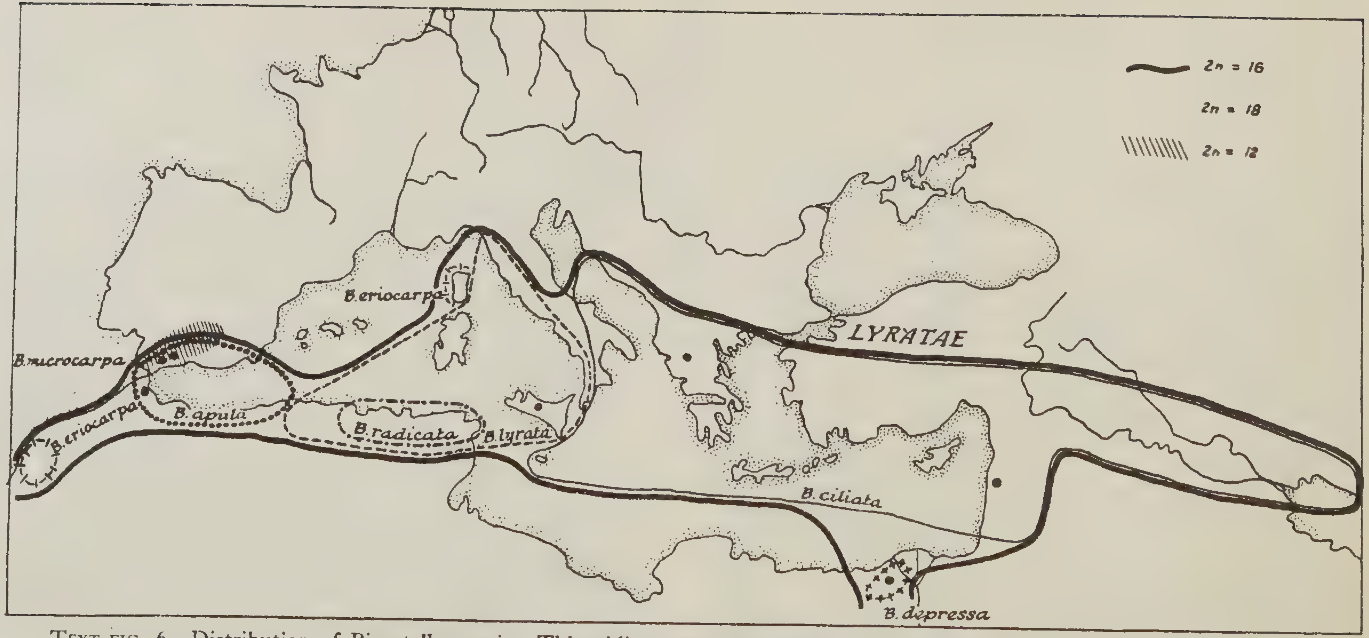
[1] I am indebted to Dr. Turrill and Mr. Gilmour of Kew for verifications of priority in nomenclature.



TEXT-FIG. 5. Phyletic scheme of subsection Lyratae after Machatschki-Laurich (1926, p. 13) with cytological data inserted.

The distribution of chromosome numbers in the Lyratae is shown in Text-fig. 5, the phyletic scheme being as before taken from Machatschki-Laurich's monograph. *B. microcarpa*, a very distinct species confined to southern Spain, has $2n = 12$ (Pl. XIX, Fig. 17). This number is unique in the genus. All the others have $2n = 16$ (Fig. 18), a number which they share with the section Iondraba. This coincidence of numbers substantiates strongly the views of the systematists as to the order of origin of the various groups. It also indicates that the diploid number 18, fundamental to the Laevigatae, is not a primitive character but derived.

The geographical distribution of the Lyratae is shown in black on the map of Text-fig. 6, the sites of cytologically investigated specimens being indicated by black spots. The sixteen-chromosomed annual stock is clearly a homogeneous one, spread on both sides of the Mediterranean and in most of the islands



TEXT-FIG. 6. Distribution of Biscutella, section Thlaspidium after Machatschki-Laurich (1926, p. 111); the subsection Lyratae in black, the subsection Laevigatae in red. The black spots represent sources of material.

except the Balearics. The limits of its extra-Mediterranean range are probably greater than the map indicates, for a form closely resembling *B. depressa* has recently been discovered in Gebl Elba in the Sudan. This extension of its known limits adds further support to the view of the systematists as to the relative age of the subsection, a view which was initially based on its prevalence in the Mediterranean islands. On the other hand, the detection of polyploidy in the Laevigatae makes the distribution of this group an even more striking contrast than was at first supposed. As the red symbols on the map indicate, the diploid stock of the Laevigatae is still limited by physical obstacles far less extreme than those which the Lyratae have surmounted. The effect of the Strait of Gibraltar may be quoted in illustration; *B. apula* of the Lyratae is on both sides of it, but all the known species of Laevigatae are confined to the northern side, for it should be noted that the only African species, *B. suffrutescens* and *B. sempervirens*, have been excluded from the Laevigatae by some systematists (e.g. Malinowski) and their true affinities cannot be decided without cytological evidence. Even more striking is the effect of the Alpes Maritimes. The complete exclusion of diploid Laevigatae from Italy, in spite of their very considerable advances to the north, can only be attributed to the narrowing of the coastal strip of land where these mountains come down to the sea. That such a barrier should be so effective argues strongly in favour of relative youth for the sub-section.

Evolution in the genus Biscutella may thus be summed up as follows. Xerothermal plants with sixteen chromosomes have existed in the Mediterranean regions possibly since the Tertiary. The oldest stock among the existing types is the section Iondraba, the subsection Lyratae of the section Thlaspidium being a later development. A still later derivative is the perennial subsection, the Laevigatae. This appears to have originated in Spain in the mid or late Quaternary, and it has migrated northwards to colonize territory more temperate in character. Following the rivers, it has circumvented the Alpine massif via the north, and it reached Austria and possibly Roumania in the last interglacial period. From it have developed a number of species several of which, for the first time in the genus, are montane or truly alpine. One of these is hexaploid and probably reached Santander by way of the Pyrenees. Another is tetraploid and appears to have entered the Alps from the west or south-west. This form has spread very rapidly in post-glacial times and now extends over most of the Swiss and Austrian Alps. A third and very pronounced alpine is the diploid form 'var. *mollis*' whose advent to its present stations in Austria is provisionally dated as glacial (i.e. the last glaciation). In the less-known Spanish flora there are others, e.g. *B. glacialis* Jord. (Manton, 1932). Developments in France cannot be correlated exactly with those in central Europe, but the endemics *B. arvernensis* and *B. Lamottii* are undoubtedly recent forms adapted to mountain scree and volcanic ash hills respectively. All these species of Laevigatae except the polyploids appear to be evolving by genetical rather than by cytological changes. All, as far as is known, are still inter-fertile, though hybrids involving the polyploids are naturally highly sterile. There is no detectable change in chromosome homology in any of the forms studied. The only other known event in their history is the partial obliteration of the diploids occupying the northern route which leaves them at the present day as relicts in Germany and Czechoslovakia.

General Biological Inferences

The cytological treatment of the problem of *B. laevigata* thus leads to an unravelling of some of the wanderings and changes experienced by an evolving group in the course of a geological period. The story is inevitably incomplete; but it reveals very clearly how much the present is the child of the past, and it provides direct evidence of evolution in a way which is only rarely possible. Some of the more general aspects of this story are worth further comment.

In the first place, it is clear that polyploidy, for all its conspicuousness, is of only subordinate importance. It has occurred at least twice and it may well occur again, the plants are effective and vigorous colonizers which spread more rapidly than their diploid relatives, but there is no reason to suppose that they are anything other than blind offshoots. Their slight instability in chromosome numbers may suggest, at first, the possibility that their descendants might stabilize out on new numbers not of the polyploid series, in the way recently indicated in another genus of Crucifers, Nasturtium (Manton, 1935), but closer inspection shows that any such tendency is likely to be inhibited by the self-sterility of the species. Even were this not so, it is quite clear that the genus reproduces in miniature what has already been demonstrated in the family (Manton, 1932). Cytological changes of major evolutionary import are those of a low numerical order. The change in one chromosome in the haploid set of eight was clearly the beginning of a new sequence of development. There is no evidence to show that such an effect has ever, in this family, ensued from a similar change imposed on a basic chromosome number as high as thirty-six or fifty-four. The polyploids are thus not likely to do more than climb further up the ladder on which they have started. Search could profitably be made in the Pyrenees to ascertain if this has already happened, for there is evidence that polyploids of a higher order than $6n$ may actually exist. Among the hexaploid seedlings already referred to one was not hexaploid but nonaploid (its chromosomes are shown in Pl. XIX, Fig. 20). This plant is vigorous, very 'gigas' in all its parts, but loath to flower under Manchester conditions: it has therefore not yet been further investigated. It is here quoted in passing only because the seed was found in nature on a hexaploid female parent and must denote either the functioning of an unreduced hexaploid gamete or the existence of plants in as high a state of polyploidy as $12n$. A hexaploid chimaera with $12n$ tissue has also been described in another connexion (Manton, 1935): there is thus every reason to expect that a $12n$ individual will be produced in the future, if it is not already there. But the conclusion seems inescapable that the more polyploid they become the less is the likelihood of these plants producing anything fundamentally new, hence the opinion of their relative unimportance, in spite of their conspicuousness.

Greater evolutionary importance must be assigned to genic mutation, and these must be inferred to have taken place without other cytological changes. This is important, for while blind mechanical forces can be shown to govern the course of change where this is cytologically determined (as in advancing polyploidy) the successful establishment of new genetical types in nature at once forces one to consider Adaptation, in a strictly Darwinian sense. The causes of genic mutations are remote and inscrutable and they must be assumed to vary greatly in the nature of their morphological expression. Many of the details which separate the species and subspecies concern characters such as hairiness, shape of leaves, size of parts, &c., which are strongly suggestive of blind chance in the nature of mutations. Nevertheless, it is abundantly clear that the salient changes of a constructive kind have actually been the adaptive ones. The two characters of perennial habit and root budding illustrate this, and the extent of their influence can only be attributed to Natural Selection.

Some insight into the mode of working of natural selection can be obtained from another series of facts. Of the new forms of diploid Biscutella whose origin has in part been traced, the most distinct morphologically are 'var. *mollis*' and *B. Lamottii*. The first has already been discussed as an alpine. The second is a most interesting plant with a very restricted and peculiar range. It is confined to the Central Plateau of France, and within this area it is only known from certain hills of a most extreme ecological type. These hills are merely piles of volcanic cinders which are only partially covered with soil and vegetation. The most conspicuous and successful of the plants forming this vegetation is *B. Lamottii*. It does not occur elsewhere. In both these cases it must be assumed that the new form originated from the more generalized type of the genus, present elsewhere in the same district but *not on the actual site*. This relation between the parental type and a successful innovation is no exception. Exactly the same thing can be deduced for the other new endemic *B. arvernensis*, or indeed, for the polyploid forms themselves. In spite of their diversity in mode of origin 'success' in all these forms has the common peculiarity that it does not appear to have involved them in competition with a parent on its own ecological station. Evolution has been effected not by closer and closer adaptation to one set of conditions but by changes of such a kind that the new form is able effectually to occupy a habitat not suitable to the parent in its original state but sufficiently near geographically to be accessible.

This fact, that the environment has acted in this genus, not necessarily as a cause, but very definitely as an opportunity, for the independent establishment of new hereditary types seems to be one of the most interesting results of the present study. It is a conclusion which invites comparison with other organisms and discussion of more general topics. It is felt, however, that in the present state of cytogenetics it is preferable to limit discussion to elucidation of the facts for each case independently. This account of Biscutella may thus appropriately close with the remark that the genus would certainly afford profitable material for further study by an ecologist or a geneticist.

SUMMARY

1. The diploid chromosome number 18 previously reported for three subspecies of '*B. laevigata* L.' (Manton, 1934) has been shown to be shared by all other available species of the subsection Laevigatae and by all the remaining subspecies of '*B. laevigata*' in which it was expected to occur. The list of these diploids now known is: '*B. laevigata* subspp. *alsatica*, *kerneri*, *subaphylla*, *gracilis*, *tenuifolia*, *guestphalica*, *austriaca* and var. *mollis*' (nomenclature of Machatschki-Laurich, 1926) from central Europe; *B. mediterranea* Jord., *B. coronopifolia* L., *B. controversa* Bor., *B. arvernensis* Jord., *B. Lamottii* Jord., *B. divionensis* Jord. from France; *B. tomentosa* Lag. and *B. glacialis* Jord. (Manton, 1932) from Spain.

2. As far as at present known, these species are not separated by sterility barriers, and among twenty-three different hybrids no trace of aberration at meiosis could be detected. This suggests that the species tested are all relatively recent and evolving by genetical rather than by cytological means. It is not certain, without more evidence from Spain, whether this is true for the whole subsection, but there is a strong expectation of uniformity.

3. Migration of the diploid stock has been from the east of Spain northward. Its progress along the Mediterranean coast has been stopped by the Alpes Maritimes, but it has circumvented the Alpine massif via the rivers to the north, and its arrival in Austria is provisionally dated as the last interglacial period. Its present status in several of its more northerly localities (in Germany and Czechoslovakia) appears to be that of a relict. In Austria a very marked diploid Alpine 'var. *mollis*' has been produced whose advent to its present rather local stations is provisionally dated as glacial, i.e. the last glaciation.

4. Of the tetraploid population characteristic of the Alps, on which interest has principally been centred, '*B. laevigata* subsp. *illyrica*' Mach. Laur. is still missing, but study of meiosis in both 'subsp. *longifolia*' and 'subsp. *lucida*' has shown quadrivalent groups in sufficient number to make these tetraploids indistinguishable cytologically from autopolyploids. This does not preclude their being genetically heterozygous, which appears to be the case.

5. The effect of multivalent pairing in producing a proportion of unbalanced gametes has been traced in both sexes, thereby explaining the occurrence of unbalanced individuals previously reported in the wild populations.

6. Within limits consequent on self-sterility the tetraploids can be combined with any of the available diploids to give triploids.

7. Meiosis in eight kinds of triploid hybrids, representing combinations of 'subsp. *longifolia*' with four diploid subspecies of '*B. laevigata*' and with four of the French species, showed that all, irrespective of origin, can form trivalents readily. It is therefore not possible to trace the parentage of the tetraploid by cytological means.

8. The only genetical criterion so far available is the occurrence of the morphological character of root budding often shown by tetraploids in cultivation. Among diploids, in Nature, it has so far only been recorded in *B. Lamottii*, *B. arvernensis*, and possibly *B. brevicaulis* Jord. An affinity between the tetraploid stock and some of the species from France is therefore probable, and it is suggested that the original tetraploid entered the Alps from the west or south-west.

9. A less complete investigation of hexaploid specimens from Spain has shown that they form multivalents, up to hexavalent, at meiosis with again the production of some unbalanced gametes. Hexaploids and tetraploids are inter-fertile and the pentaploid hybrids resulting form pentavalents. The hexaploid is also fertile with some diploids. From the morphology of the 'pseudotetraploids' thus produced it is inferred that the genetical constitution of the Spanish polyploid is not identical with that of the Alpine tetraploids.

10. A nonaploid individual with eighty-three chromosomes is recorded from among the hexaploid seedlings.

11. The following recommendations on changes in nomenclature to incorporate the above facts in the taxonomic system are made:

(*a*) that 'var. *mollis*' should again be accorded specific rank, in which case its name should be *B. minor* Jord.

(*b*) that the tetraploids (= subspp. *longifolia*, *lucida*, *illyrica* (?) and varieties) be collectively regarded as a separate species. This species should retain the name *B. laevigata* L. since the Linnean type specimen was a tetraploid of the *longifolia* type from the Alps north of Verona.

(*c*) that among the remaining diploids previously associated with *B. laevigata* priority of name goes to *B. austriaca* Jord., but the possibility is not excluded that further genetical evidence might show the desirability of reconsidering specific boundaries here.

(*d*) that separation of the high polyploids of northern Spain is probably desirable, though these are still incompletely known.

12. Comparative reference to the rest of the genus, particularly to the subsection Lyratae, has shown that these are characteristically annuals with a lower chromosome number ($2n = 16$ in *B. apula* L., *B. lyrata* L., *B. ciliata* D.C., *B. depressa* Will.; $2n = 12$ in *B. microcarpa* D.C.). Both the Lyratae and the other section Iondraba show a geographical range suggestive of greater antiquity than the Laevigatae.

13. In assessing factors of evolutionary importance, predominance is given to cytological changes of a low numerical order and to genetical changes (presumably mutations) of a kind such as can make possible the establishment

of adaptive relations to habitats other than that of the parent stock. The polyploids are regarded as highly successful but relatively unimportant and blind offshoots.

ACKNOWLEDGEMENTS

I am indebted to several correspondents for gifts of material. They include Dr. Wein of Nordhausen for subsp. *tenuifolia*, Fräulein Wallner of Vienna for subsp. *austriaca*, Miss Legge of Manchester for *B. divionensis*, and Mr. Tutin of Plymouth for *B. tomentosa*. For technical assistance I owe thanks to Miss Colson for help in details of staining in haematoxylin, to Dr. Heitz and Dr. Philp for details of the Heitz and McClintock acetocarmine techniques, to Mrs. Palmer for some of the chromosome counts, and to Mr. Gilmour of Kew for help in setting up the hybrids and in many other ways. Finally, very special thanks are due to Professor Weiss and to the authorities of Kew and the staff of Manchester University Experimental Grounds for the culture of the plants.

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EXPLANATION OF PLATES XIX and XX

Illustrating Dr. I. Manton's paper on 'The Problem of *Biscutella laevigata* L. II. The Evidence from Meiosis.'

PLATE XIX

All photographs are from haematoxylin preparations at a magnification of 2,000, except Fig. 7, which is magnified 1,000.

Fig. 1. Portion of first metaphase of pollen mother-cell of tetraploid subsp. *longifolia* (plant 18.16). A pair and a quadrivalent in focus, a trivalent and a single chromosome out of focus, to the left. Sectioned material fixed in modified 2 BD.

Figs. 2–4. Three focal levels of one uncut nucleus at diakinesis in sectioned material of tetraploid subsp. *longifolia* (plant 18.10). Full analysis in Text-fig. 2. Fig. 2 focused on a trivalent and a single. Fig. 3 shows rings of four clearly at top and bottom, other rings less well placed in the centre. Fig. 4 a quadrivalent and two pairs.

Figs. 5 and 6. Portions of two cells at diakinesis in a pentaploid hybrid (No. 36*a*) of parentage-hexaploid (No. 43.4) ♂ × tetraploid subsp. *longifolia* (No. 33.12) ♀, showing a quadrivalent (Fig. 5) and a pentavalent (Fig. 6). Sectioned material fixed in 2 BE with both osmic and acetic acids doubled. Other figs. from this plant in Pl. XX, Figs. 30–2.

Figs. 7 and 8. Diakinesis from sectioned material of diploid subsp. *alsatica* (plant 35.11). Fig 7. A group of cells. × 1,000, Fig. 8, a single bivalent. × 2,000.

Figs. 9–10. Two focal levels of one nucleus of hexaploid (plant 43.6). Fig. 9 shows a hexavalent ring, Fig. 10 a quadrivalent ring. Fixative 2 BE pretreated with Carnoy's fluid.

Fig. 11. Metaphase of second division in the same hexaploid (43.6) showing $3n+1$, i.e. 28 chromosomes. The somatic number of the plant was $6n+1$, i.e. 55.

Fig. 12. Second division in tetraploid subsp. *longifolia* (33.15) with all chromosomes visible, showing uneven distribution. The upper plate shows 17, the lower 19. From a smear fixed in 2 BD diluted with half its volume of water, stained in haematoxylin.

Fig. 13. Polar view of first metaphase of diploid species *B. controversa* from the Auvergne showing $n = 9$. Sectioned material fixed 2 BE with doubled osmic.

Fig. 14. An exceptional cell in a root of diploid species *B. divionensis* from the neighbourhood of Dijon, showing an incipient chimaera; 18 doubled chromosomes visible. Fixed 2 BE.

Fig. 15. Root of a triploid (No. 88*b*) with an extra chromosome, 28 visible, from the cross *B. coronopifolia* ♂ × tetraploid subsp. *longifolia* ♀. The tetraploid female parent (plant 21.1) had a regular chromosome number (36). This hybrid therefore demonstrates the production of unbalanced female gametes (cf. Fig. 12 for male). Fixative 2 BE.

Fig. 16. The formation of an unreduced gamete in a spontaneously produced triploid. All 27 chromosomes in one plate at the second division.

Fig. 17. *B. microcarpa* from south Spain, root with 12 chromosomes. Fixative 2 BE.

Fig. 18. *B. ciliata* from Jerusalem, root showing 16 chromosomes. Fixative 2 BE.

Fig. 19. *B. tomentosa* Lag. (= *B. montana* sensu Mach.-Laur.) from Gibraltar, root showing 18 chromosomes. Fixative 2 BE.

Fig. 20. A nonaploid individual among the hexaploid seedlings from Spain, root with $9n$, i.e. 81 chromosomes.

PLATE XX

All photographs are from permanent acetocarmine smears of pollen mother-cells.

Fig. 21. First metaphase, diploid hybrid (No. 109*b*), *B. laevigata* var. *austriaca* ♂ × *B. coronopifolia* ♀ showing nine pairs of chromosomes. × 1,500.

Fig. 22. First metaphase, diploid hybrid (No. M65) var. *mollis* ♂ × *B. controversa* ♀ showing nine pairs of chromosomes. × 1,500.

Fig. 23. First anaphase, diploid hybrid (No. 103*b*) of subsp. *tenuifolia* ♂ × *B. arvernensis* ♀ showing regular disjunction of 9+9 chromosomes. × 1,000.

Fig. 24. First metaphase, triploid hybrid (No. 88) of *B. coronopifolia* ♂ × tetraploid subsp. *longifolia* (No. 21.1) ♀, four or five trivalents, pairs and singles. The doubtful trivalent is the third one up from the bottom, in which the large chromosome on the left is probably not attached. × 1,500.

Fig. 25. First anaphase, triploid hybrid (No. 3*b*) of tetraploid subsp. *longifolia* × subsp. *alsatica* to show general features of triploid meiosis. Six unpaired chromosomes lag on the spindle and have divided. At the poles are 12 chromosomes on the left and 9 on the right representing the separation of six pairs and three trivalents; all these show the split in preparation for the next division. × 1,000.

Fig. 26. The same plant as Fig. 25 at the end of the second division showing the inequality in distribution of chromosomes increased by the further lagging of the split singles. The four nuclei of the tetrad show 12, 13, 15, 14 chromosomes in the order from left to right. × 1,000.

Fig. 27. Diakinesis in the tetraploid subsp. *lucida* (plant 20.1), the same individual whose

somatic chromosomes were photographed in Fig. 16 of the first paper (Manton, 1934). Six to eight quadrivalents, the rest in pairs. Full analysis in Text-fig. 3. × 1,500.

Fig. 28. Regular segregation of 18 + 18 chromosomes, for comparison with Pl. XIX, Fig. 12, at the first anaphase in a cell of tetraploid subsp. *longifolia* (plant 18.1). × 1,000.

Fig. 29. Unsquashed cell in acetocarmine, showing 18 chromosomes at the second division in the 'pseudotetraploid' hybrid of the diploid species *B. coronopifolia* ♂ × hexaploid (No. 43.6) ♀. × 1,000.

Fig. 30. Chromosome pairing in pentaploid hybrid (No. 36*a*), of hexaploid (No. 43.4) ♂ × tetraploid subsp. *longifolia* (plant 33.12) ♀, the hybrid with 5*n* − 1, i.e. 44 chromosomes, in all valencies from 1 to 5. Full analysis in Text-fig. 4. × 1,500.

Fig. 31. First anaphase in the same pentaploid individual for comparison with corresponding stage in diploid, triploid, and tetraploid (Figs. 23, 25, and 28). Four unpaired chromosomes lag and are dividing on the equator, while 20 paired chromosomes have gone to each pole (one, destined for the left pole, has not yet separated). × 1,500.

Figs. 32*a* and *b*. Squashed tetrad from the same pentaploid individual for comparison with triploid (Fig. 29). The four nuclei with 21, 23, 22, 21 chromosomes, in order from left to right. The two figures are part of a single photograph, but they lie rather far apart in the original, therefore a piece has been cut out from between them to avoid waste of space. × 1,000.

Fig. 33. Early anaphase of triploid hybrid (No. 33*a*), of *B. mediterranea* ♂ × tetraploid subspecies *longifolia* (No. 27.2) ♀, showing six trivalents, three pairs, and three singles. × 1,500.

Fig. 34. Chromosome pairing at metaphase of triploid hybrid (No. 28) incorporating subsp. *alsatica* (No. 1.1) ♂ with tetraploid subsp. *longifolia* (19.7) ♀. Seven to eight trivalents; the only doubtful group is the second from the right at the top, in which the superposed chromosome, looking dark, may or may not be attached. × 1,500.

Fig. 35. Chromosome pairing at metaphase in triploid hybrid (No. 61) incorporating *B. Lamottii* (FIa) with tetraploid subsp. *longifolia* (No. 21.1); seven trivalents, two pairs, and two singles very clear. × 1,500.

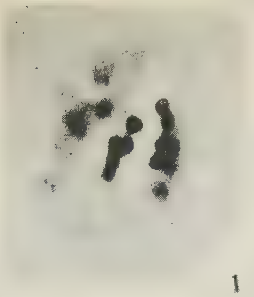
Fig. 36. Chromosome pairing at metaphase in triploid hybrid (No. 128) incorporating subsp. *austriaca* with tetraploid subsp. *longifolia* (No. 21.1); seven trivalents, two pairs, and two singles (one pair and one single make the second group from the left, the other pair and single make the third group from the right). × 1,500.

Fig. 37. Chromosome pairing at metaphase in triploid hybrid (No. 100) incorporating subsp. *kerneri* with tetraploid subsp. *longifolia* (No. 18.7); seven trivalents, two pairs, and two singles. The two pairs are together in the middle; the two singles are at the bottom right. × 1,500.

Fig. 38. Chromosome pairing at metaphase in triploid hybrid (No. 85*b*) incorporating *B. controversa* with tetraploid subsp. *longifolia* (No. 21.4), showing six trivalents, three pairs, and three singles. × 1,500.

Fig. 39. Chromosome pairing at metaphase in triploid hybrid (No. 130) incorporating subsp. *tenuifolia* with tetraploid subsp. *longifolia* (No. 19.6), showing seven trivalents, two pairs, and two singles. × 1,500.

Fig. 40. Part of a squash at diakinesis in the hexaploid (plant 43.6), showing a zigzag hexavalent and a ring of four. × 1,500.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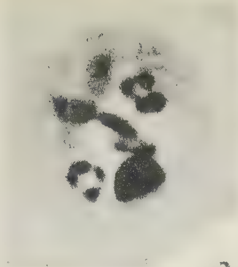
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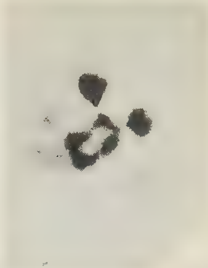
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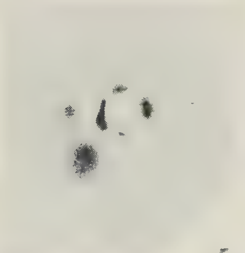
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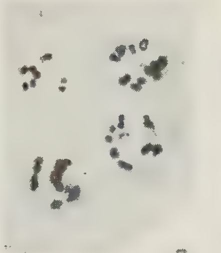
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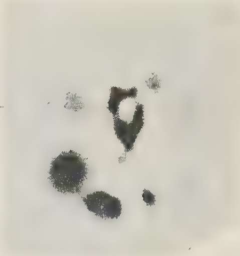
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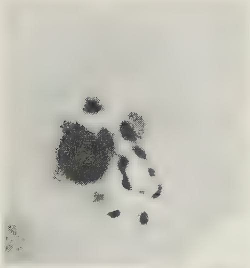
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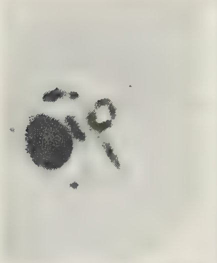
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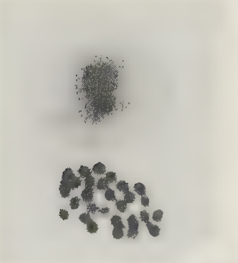
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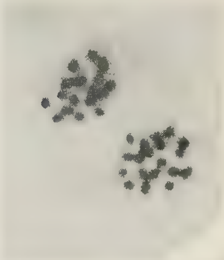
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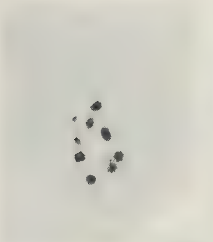
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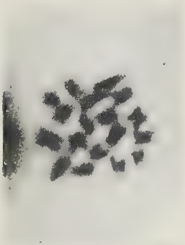
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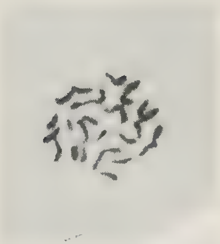
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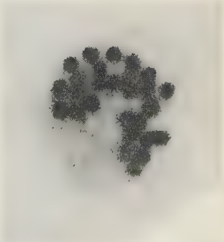
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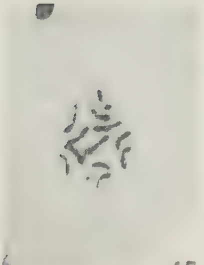
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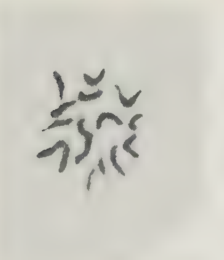
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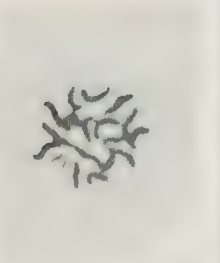
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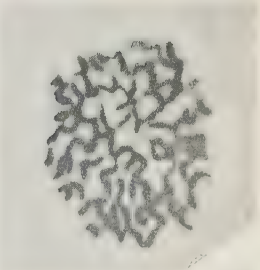
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MANTON — BISCUTELLA LAEVIGATA.

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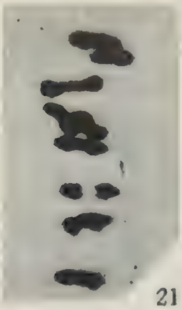
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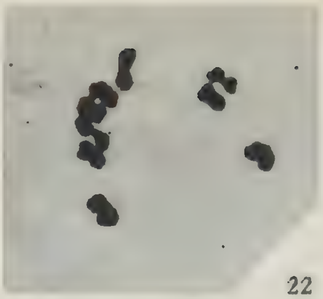
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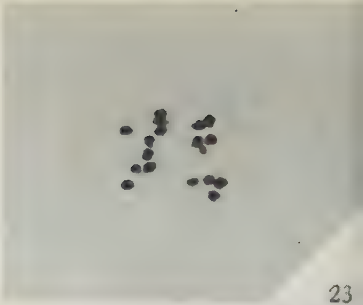
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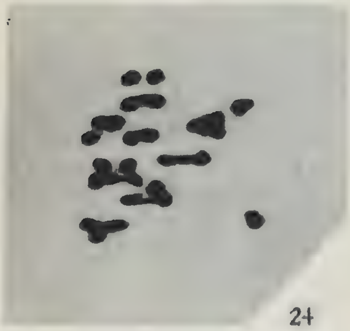
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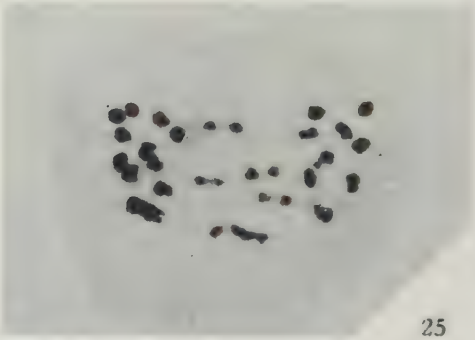
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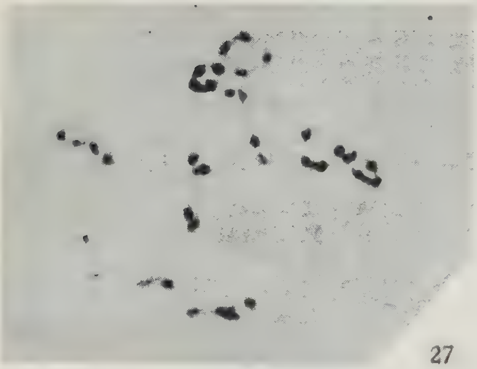
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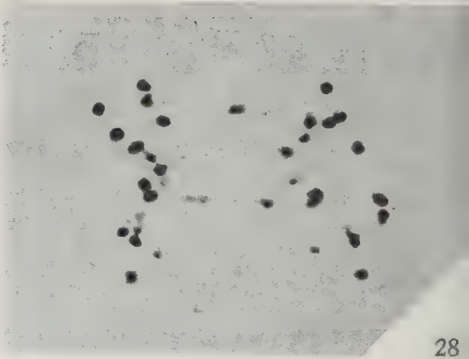
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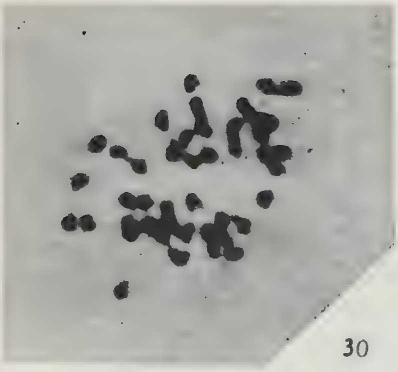
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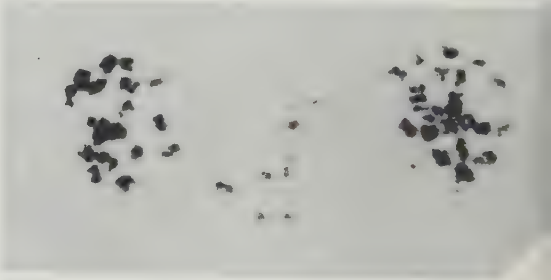
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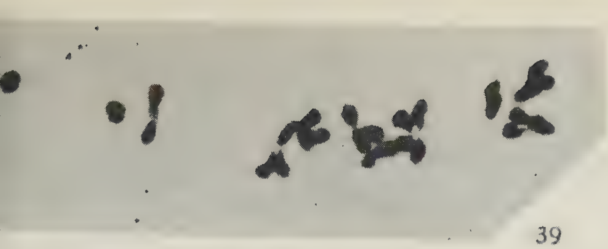
MANTON — BISCUTELLA LAEVIGATA.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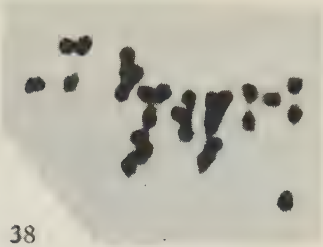
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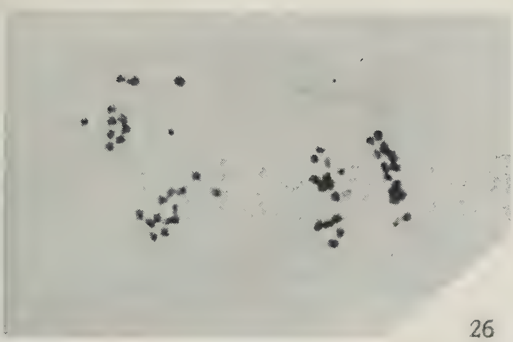
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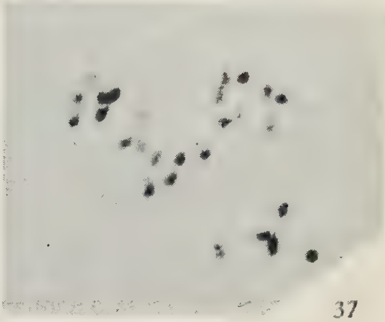
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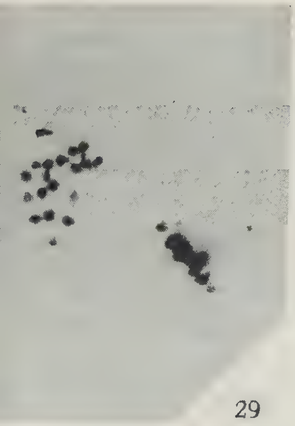
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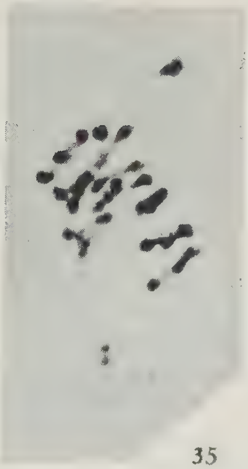
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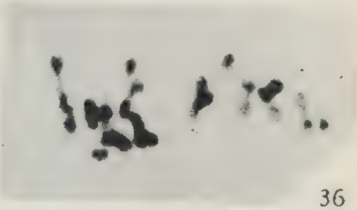
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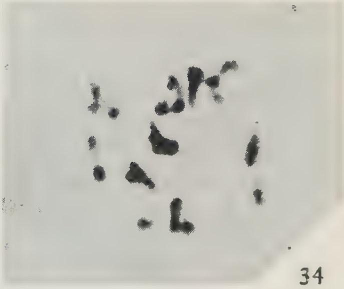
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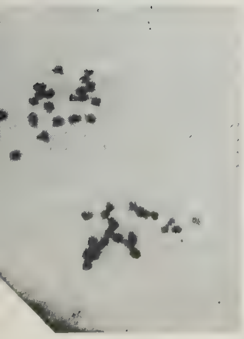

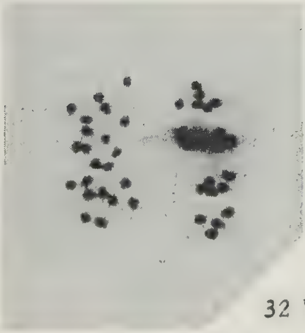
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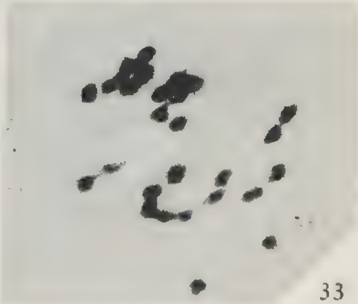
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Spermothamnion Snyderae Farlow, a Floridean Alga bearing Polysporangia

BY

KATHLEEN M. DREW (MRS. BAKER)

(*From the Cryptogamic Research Laboratory, the University of Manchester*)

With Plate XXI and twelve Figures in the Text.

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I. INTRODUCTION

Spermothamnion Snyderae Farlow is an alga belonging to the Ceramiaceae and calls for special attention as it is one of the Florideae which bear sporangia containing more than four spores, in place of the usual tetrasporangia. The position and development of these sporangia of *S. Snyderae* suggest that they are the morphological equivalent of the tetrasporangia of other Florideae. A cytological investigation was undertaken in order to see whether the nuclear history would further support this assumption.

The sporangium initial of the majority of the Floridean algae is uninucleate, and after meiosis four uninucleate spores are formed. This is known to be so even in the case of those species which have multinucleate somatic cells, e.g. *Spermothamnion Turneri* (Mert.) Aresch. (Drew, 1934). On the other hand, the young sporangia of *S. Snyderae* are multinucleate, and, as the following pages show, all of the nuclei present in the initial undergo meiosis, resulting in a sporangium containing several spores, i.e. a polysporangium. In these sporangia the total number of spores is a multiple of four, and the polysporangium can be considered therefore as a compound tetrasporangium.

The nuclear history of the polysporangium forms the main subject of this paper, but the account of the life-history is completed by a description of the sexual reproduction.

A number of abnormalities, particularly among the sporangia, some of greater theoretical importance than others, have been noted in the course of this work, and these are described briefly in smaller print at the end of the appropriate section of the paper.

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II. MATERIAL AND METHODS

The only records of *Spermothamnion Snyderae* Farlow come from the Pacific Coast of North America, where, according to Farlow (Farlow, 1899), it occurs between Santa Cruz and San Diego. Since the date of Farlow's description, however, other collectors have extended the northern limit of its range and the material used in this investigation was collected at Bolinas, a few miles north of San Francisco.

S. Snyderae is a branched filamentous alga, growing in tufts, 2 to 2½ in. long. It is found on and between small sand-covered rocks and appears to bind the sand, building up miniature dunes.

A mixture of 100 c.c. of 70 per cent. alcohol and 6 c.c. of 40 per cent. formaldehyde was employed as a fixative, and fixings were made on the shore at various times during the day and night. This was possibly unnecessary, as division figures have not been difficult to find.

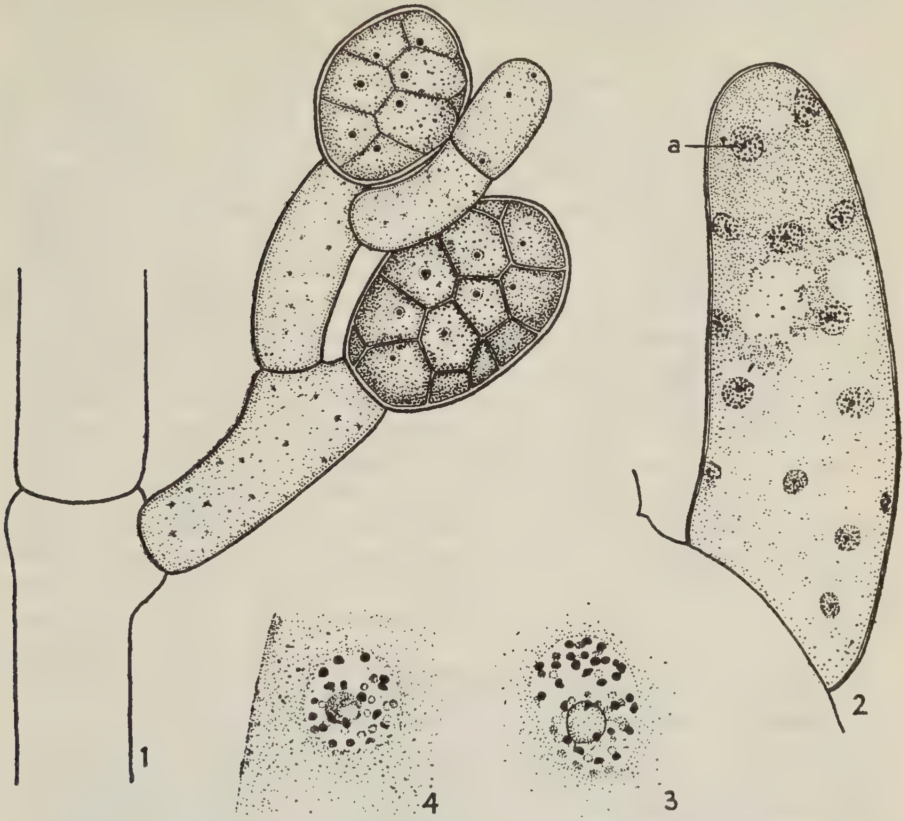
The filaments have been stained with brazilin and mounted whole, but owing to their large size it has been impossible to prevent some crumpling of highly vacuolate cells. It is of interest to note that this very rarely interferes with the structure of the nuclei. Essentially the same method of staining and mounting as that used for *Spermothamnion Turneri* (Drew, 1934) has been employed for this species. Instead of passing the material from absolute ethyl alcohol through alcohol and xylol mixtures, however, it has been taken gradually into isopropyl alcohol and from that into mixtures with xylol. It has been necessary to make the passage through the dehydrating and clearing solutions a very slow one. Both canada balsam and Flatters and Garnett's Neutral Mounting Medium have been used for mounting.

A Watson Holoscopic Immersion Condenser and critical illumination from a Pointolite Lamp have been used, as without such refinements observations are difficult and possibly unreliable.

III. CYTOLOGY OF THE SPORANGIUM

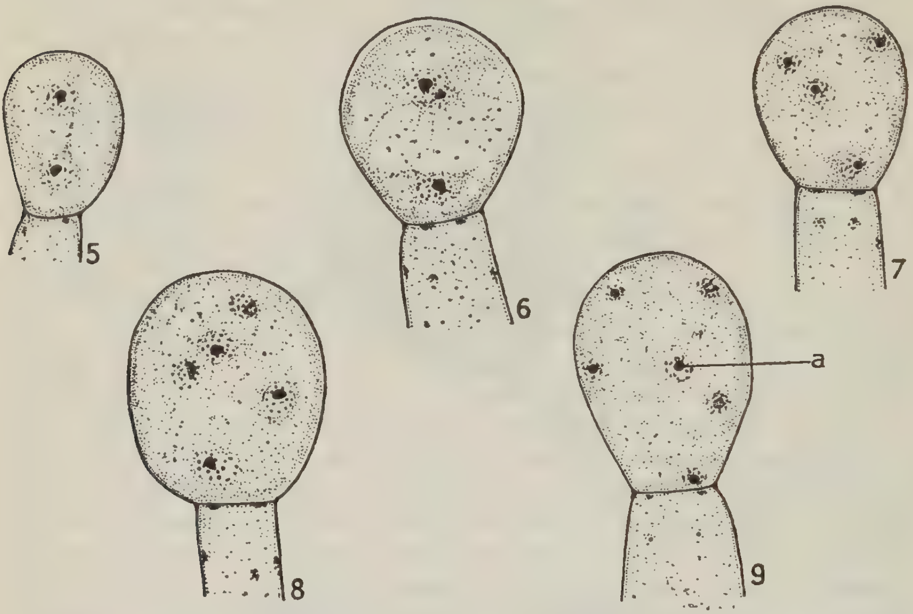
The sporangia occur in clusters along the main filaments and terminate branchlets, which are irregularly cymosely branched. Various stages of development are to be found in any one cluster (Text-fig. 1). The apical portion of any branchlet may be transformed into a sporangium initial. When this is about to happen an increase in the density of the cytoplasm in the apex of the cell occurs prior to the formation of the transverse wall and this distinguishes the separation of a sporangium initial from an ordinary vegetative division. In addition, a plate of cytoplasm is laid down across the central vacuole, so that the portion destined to become the sporangium is quite distinct even before the dividing wall is formed (Plate XXI, Fig. 1). The size of the newly formed sporangium initial varies considerably, and this, no doubt, explains the varying size of the mature sporangia. The sporangium initial begins to increase in size directly it is formed, particularly in the transverse diameter, so that by the time the first nuclear division takes place it is much more spherical than it was originally. After this division it continues to increase in size until the spores are formed (Text-fig. 1). The mature sporangia are oval or circular in longitudinal section.

Like the other somatic cells of *S. Snyderae*, the cell which gives rise to a sporangium initial is multinucleate. Preceding the formation of the sporangium, the nuclei divide. Text-fig. 2 shows such a cell with the nuclei in prophase and Text-fig. 3 the nucleus of that cell, marked *a*, in detail. Sixty-four chromosomes are distinguishable in the nucleus figured, this being the diploid number and that found in the cells of sporangia-bearing plants. The number of nuclei in the apex of the cell decreases considerably after this nuclear division. No degenerating nuclei have been seen, but there is some



TEXT-FIGS. 1–4. Fig. 1. Branchlet bearing three polysporangia. One is mature, the furrows delimiting the spores are forming in the second, and the third has just been cut off from the stalk-cell. Note that the youngest sporangium contains three nuclei. × 263. Fig. 2. Cell from which sporangium will be cut off, with nuclei in prophase. Only nuclei on upper side of cell shown. × 630. Fig. 3. Nucleus, marked *a* in Fig. 2, at greater magnification. Diploid. Sixty-one chromosomes figured and, in addition, three behind nucleolus. × 2,590. Fig. 4. Nucleus from somatic cell of female plant for comparison with Fig. 3. Thirty-two or thirty-three chromosomes present, the doubtful one being behind the nucleolus. × 2,590.

evidence, on the other hand, that the decrease is brought about by a movement of nuclei towards the base of the cell. A young sporangium may contain anything from two to nine nuclei, but three and four are the numbers much the most commonly found. The higher numbers do not occur as often as the lower ones, which are shown in Text-figs. 5–9. All of the nuclei in the sporangia figured are either approaching or in late diakinesis. There is not the slightest evidence of any nuclear degeneration in the young sporangium, and all of the nuclei present enter the prophase of the heterotype division. The prophase stages of this division appear to resemble closely those described for *Spermothamnion Turneri* (Drew, 1934), to which the reader is referred for full details. There is a long, thin, meandering thread (or possibly threads) of chromatin in the nucleus in the early stages, and from this condition the nucleus passes into synizesis. This is a very distinctive stage and one of considerable duration, judging from the numbers seen. A sporangium containing four nuclei at synizesis is shown in Pl. XXI, Fig. 2. After synizesis the chromatin thread is once more evenly distributed throughout the nuclear area but it is shorter and thicker and of even width. This stage is followed by one where there are separate and distinct lengths of chromatin which, judging from their number, are the gemini. After the appearance of the gemini the nuclear area, the nucleolus, and the gemini themselves enlarge. From then until late diakinesis the gemini do not stain very readily. When



TEXT-FIGS. 5–9. Sporangia prior to the first nuclear division, containing 2, 3, 4, 5, and 6 nuclei respectively. The nuclei are either approaching (Figs. 7 and 9) or in late (Figs. 5, 6, and 8) diakinesis. ×350.

the gemini first appear they are irregular knotted threads, and later the doubleness of these threads becomes evident by diamond-shaped endings or forked ends. A slightly later stage is shown in Pl. XXI, Fig. 4, which represents one (*a*) of the six nuclei in the sporangium of Text-fig. 9. It should be emphasized that all six nuclei in the sporangium are in the same condition. Later the gemini shorten and thicken, the doubleness being still evident, but eventually they appear as single bodies, which are only slightly longer than wide. The three nuclei of the sporangium of Fig. 3, are in this condition. The nuclei are quite near the periphery of the sporangium, one towards the base (*a*) and two in the apical region (*b* and *c*). These three nuclei are figured separately at a greater magnification (Figs. 3, *a*, *b*, *c*). There are thirty-two gemini[1] present in each, but in the drawing of the basal nucleus one is not shown (Fig. 3*a*) as it is under the nucleolus. The same applies to one chromosome pair of nucleus *b* (Fig. 3*b*) and four of nucleus *c* (Fig. 3*c*). In addition to the gemini there are other structures *p* present in two of the nuclei (Figs. 3*a* and *c*) Without the evidence provided by these prophase stages, which are typical of a meiotic division, the presence of thirty-two 'bodies' at the stage represented in these figures is in itself proof of a reduction in the number of the chromosomes, as it is half the number present in the somatic divisions.

Additional proof is provided by filaments which have somatic nuclei as well as nuclei in sporangia in division. Two such filaments have been seen in the material examined and, as would be expected, the somatic nuclei have the diploid number of chromosomes and those in the sporangia the haploid number.

Towards the end of diakinesis the nucleolus becomes very lobed, but by the time metaphase is reached it has disappeared completely. Apart from the absence of any achromatic figure and the reduced size of the nuclear area, there is nothing of particular interest to report about metaphase. There is little to indicate whether any particular example belongs to the first or second division. Where there is an odd number of plates it is reasonably safe to presume it is the first division. On the other hand, unless a very high one, an even number cannot be taken as proof of the homotype division. In some cases where the plates number three or four, what are presumably the gemini are clearly defined and disjunction has been seen.

A small darkly staining body is frequently present at telophase or later, in the strand of denser protoplasm connecting the reorganizing nuclei, when it is often nearer one nucleus than the other. It has been seen only rarely at metaphase, and then it is immediately outside the nuclear membrane. It sometimes persists after the daughter nuclei are completely reorganized.

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[1] Although counting is comparatively easy at this stage, the presence of deeply staining bodies in the cytoplasm introduces a difficulty. These bodies are sometimes very like the gemini in size and shape, and when they occur near the nucleus it is not easy to decide whether they are in the nucleus or not as the nuclear membrane is so thin. In addition, the nucleolus is often lobed and sometimes has an irregular surface, so that small gemini are hidden. The number thirty-two is the result of studying forty nuclei in detail and agrees with somatic counts.

Having ascertained that the sporangium initial is multinucleate and that the first division of the nuclei is a reduction division, the remaining question concerns the number of nuclear divisions, a question which can be answered by inference only. In the first place, the numbers of uninucleate spores in a sporangium are most commonly twelve and sixteen, and these numbers would result from two divisions of three and four parent nuclei respectively. These, indeed, are the numbers of nuclei most commonly found in the sporangium initials, as has been stated previously. Secondly, the fact that mature sporangia containing twelve and twenty uninucleate spores occur, means that not more than two nuclear divisions have taken place. The same would apply to a sporangium with twenty-eight spores. Incidentally, sporangia containing twelve, twenty, and twenty-eight nuclei cannot arise from a uninucleate initial without irregularity in the divisions. Eight, twelve, sixteen, twenty, twenty-four, and thirty-two spores per sporangium have been seen, but judging from the numbers of nuclei in the young sporangia other numbers are possible.

Very few examples of sporangia in stages between the end of the first division and the commencement of invagination have been seen and even fewer examples of the prophase stage of the second nuclear division at a time when the chromosomes are discrete. The chromosomes at the time of the second division are about the same size as those of the somatic nuclei, but the nuclear area is considerably larger, although not quite as large as during the later stages in the prophase of the heterotype division. The chromosomes, which are haploid in number, show signs of occasional fortuitous pairing, as in the case of the nucleus figured (Pl. XXI, Fig. 5). The second division takes place at the periphery of the sporangium.

The spores are formed by invagination of the protoplasm. Prior to the appearance of the furrows, the nuclei arrange themselves over the periphery of the sporangium, the protoplasm becomes less vacuolate, and the plastids reappear. As the furrows deepen the nuclei move towards the centre of the sporangium, but the nucleus of the mature spore is centrally placed. The invagination furrows do not follow any regular geometrical pattern, but are sometimes so irregular that spores of different sizes are formed in the same sporangium.

Abnormalities. In the course of this investigation various abnormalities have been seen and will now be described briefly. Two terminal uninucleate cells have occurred, both among sporangia-bearing branchlets. Whether they are young sporangia or not is uncertain, for while their position suggests they are, their size does not.

Occasionally sporangia containing binucleate as well as uninucleate spores are produced. As the nuclei total a number of four in such cases, it seems safe to assume that two nuclei have become enclosed in one spore as the result of irregular invagination. In a very irregularly partitioned sporangium a quadrinucleate spore occurs in addition to some binucleate spores. The nuclei in this sporangium total twenty-four, again a multiple of four. In the case of a sporangium containing twenty-three uninucleate spores, the twenty-fourth nucleus is to be seen in the centre of the sporangium, surrounded by a small amount of protoplasm.

These abnormalities concern mature sporangia containing the expected number of nuclei, but others containing an irregular number also occur, if but rarely. Sporangia in the course of development and containing small and faintly staining nuclei, in addition to others which are normal, have been seen and are probably to be regarded as earlier stages of this condition, especially as they occur on the same filament.

In one collection of material five sporangia containing nuclei with aberrant chromosome numbers occur. These sporangia have other unusual features in common, one of which is the preparation of the nuclei for division, although invagination has started. The unusual number of nuclei present, the irregular shape of the nuclei, and the unhealthy condition of the sporangia are also noticeable. The nuclei of these sporangia are not all in the best condition for counting the chromosomes, but it is certain that in every case nuclei with two or even three different numbers of chromosomes occur in the same sporangium. One contains haploid and diploid nuclei; and another, in addition to diploid nuclei, one triploid nucleus. In the three others, nuclei containing forty-five to fifty chromosomes occur with either haploid or diploid nuclei, or even both. A few cases of coalescence of nuclei during the prophase of the meiotic division have been seen in sporangia on a filament bearing one of these abnormal sporangia and also known to be diploid. This coalescence of nuclei in the young sporangia would appear to provide the explanation of the unusual chromosome numbers just mentioned, but the reason for the coalescence is still obscure. Apparently this coalescence does not interfere with meiosis. The filaments bearing these abnormal sporangia bear in addition many others which have degenerated completely. In all probability, therefore, these abnormal sporangia never produce mature spores, and there seems no reason for presuming they have any bearing on the usual life-cycle.

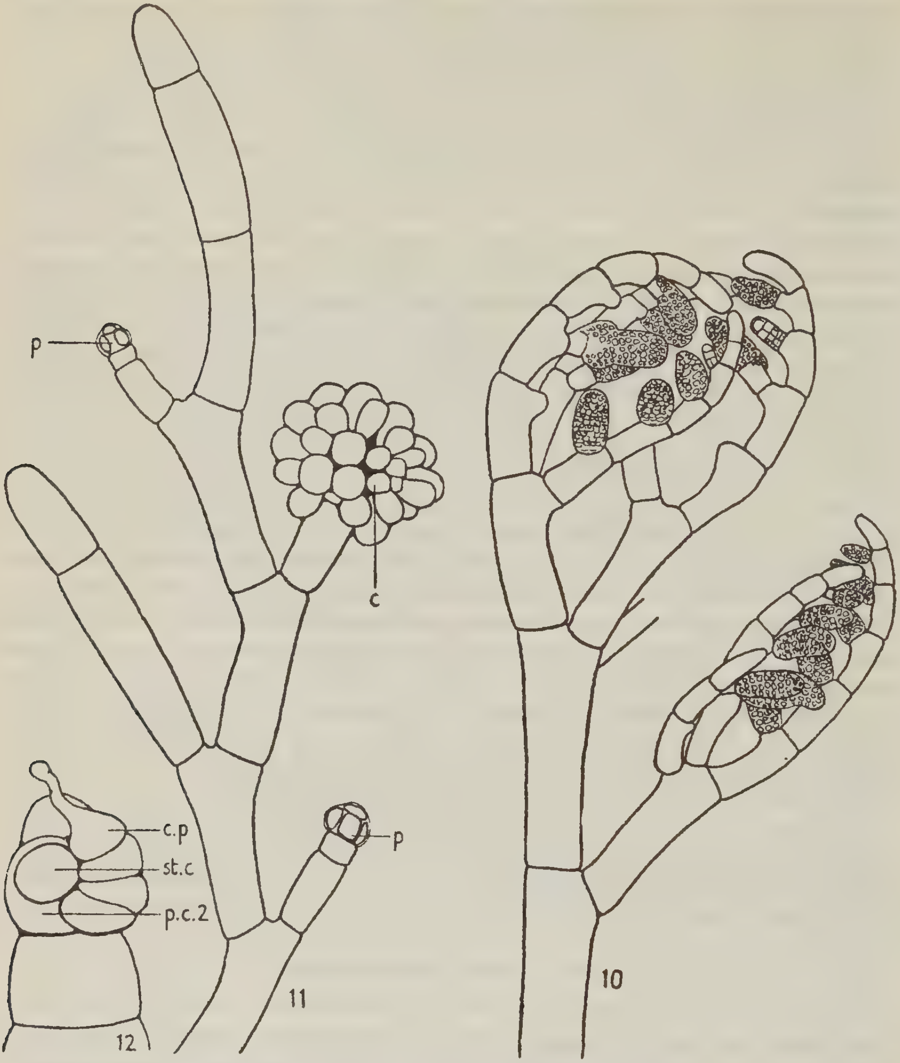
The two nuclei of the terminal cell of a branchlet have been found to contain chromosomes numbering, approximately, 128, the tetraploid number. The cell occupies the position of a sporangium and its aberrant chromosome number is probably to be attributed to the same cause as that in the sporangia just described.

IV. Sexual Reproduction

Unless in a sterile condition, sexual plants can be distinguished easily from those bearing polysporangia by the aid of a hand lens. It seems likely that *S. Snyderae* is a dioecious species as, although male and female filaments may occasionally occur in close association in one tuft, they have never been found on the same filament. One tuft may possibly be composed of several plants resulting from the germination of a group of spores.

Male plants.

Farlow (loc. cit.) does not appear to have seen the male plants, but they are neither small nor rare in the material collected in November. The filaments are rather sparsely branched in the lower region and bear a series of secund branchlets near their apices (Text-fig. 10). One, or sometimes two, secondary branchlets, which are also secund, develop on the adaxial side of each of the cells at the base of the primary branchlets. Any cell of a branchlet (excepting the apical) may give rise to one spermatangial branchlet, and this also develops on the adaxial side (Text-fig. 10). The branchlets are strongly curved, the spermatangial branchlets being on the inside of the curve. This arrangement gives a feathery appearance to the filaments.



TEXT-FIGS. 10–12. Fig. 10. Upper part of a male filament with numerous spermatangial branchlets. × 123. Fig. 11. Branch of female plant with two young procarps (*p*) and a mature cystocarp (*c*). × 123. Fig. 12. Mature procarp viewed from the side of the second pericentral cell (*p. c.* 2); *st. c.* = sterile cell; *c. p.* = carpogonium terminating carpogonial branch. × 630.

In contrast to a newly formed vegetative branchlet, a spermatangial branchlet at the corresponding stage is uninucleate (Pl. XXI, Fig. 6, *sp. br.* 1). The cell elongates and successive transverse divisions, preceded by divisions of the nucleus, take place to give a row of four to six cells. Before the last of these divisions is completed the lower segment cells have become multinucleate (Fig. 6, *sp. br.* 2). Pericentral cells are cut off progressively from each segment cell of the branchlet. Those derived from the basal cell are shorter than the basal cell itself, as the dividing wall is oblique. The spermatangial mother-cells are formed by further divisions of the pericentral cells and also from the apical cell of the spermatangial branchlet. They form a continuous layer over the whole branch, and on their free surface the spermatangia develop. Very rarely a spermatangial branch is itself branched. After the spermatia are liberated the whole branchlet drops off.

The pericentral cells and spermatangial mother-cells are uninucleate, and in all the nuclear divisions seen in cells of developing spermatangial branchlets the chromosomes are haploid in number, as in the nucleus of Fig. 6*a*. In this figure, which is that of the nucleus of the cell marked *c* in Fig. 6, a doubtful chromosome is hidden behind the nucleolus.

Female plants.

The procarps are terminal on branchlets whose branching, although somewhat irregular, is fundamentally cymose (Text-fig. 11). They develop in exactly the same way as those of *Spermothamnion Turneri* (Drew, 1934), and for illustrations of the development the reader is referred to that paper. Figures 7 and 8 on Pl. XXI illustrate stages not given in the previous paper, however. When the apical cell of a branchlet is to be transformed into a procarp, all the nuclei excepting one move to the basal end of the cell (Fig. 7). A transverse wall is then laid down separating the apical part containing the single nucleus, which has enlarged considerably by this time from the rest. The newly formed apical cell divides by a transverse wall to give two uninucleate cells (Fig. 8). The lower of these cuts off three pericentral cells, the second and third developing one on either side of the first. The second pericentral cell cuts off a small cell, the sterile cell, to the outside. The inner cell is the supporting-cell, and from it the four-celled carpogonial branch develops on the side away from the first pericentral cell. When fully developed the carpogonial branch is exactly opposite the first pericentral cell and between the second and third, the cells from which the auxiliary cells develop. Text-fig. 12 shows a mature procarp. The first and third pericentral cells are out of sight on the back side.

The haploid number of chromosomes has been seen in dividing nuclei in cells of filaments bearing procarps (Text-fig. 4), and the same number appears in the nuclear divisions of the developing procarps. Two examples are figured, one the nucleus (Fig. 8*a*) of the fertile segment of a young procarpic branch (Fig. 8), and the other the nucleus (Fig. 9*a*) of the apical cell of a three-celled carpogonial branch. There are thirty-two chromosomes in the former, but only thirty-one have been distinguished in the latter.

The nucleus of the apical cell of the procarp, the first pericentral cell, the sterile cell, and the cells of the carpogonial branch (excepting the carpogonium) all become multinucleate, but there is no further division of the cells.

After fertilization the supporting cell divides by an oblique wall into a smaller basal cell and a much larger cell, the auxiliary cell. A similar division takes place in the third pericentral cell. The nuclei in the basal cells may divide, but the cells remain undivided.

Fertilization has not been seen, but the procarp of Pl. XXI, Fig. 10, shows the changes which take place after fertilization and also provides the evidence of nuclear fusion. The carpogonial branch (*cp. br.*) is on the near side of the procarp and its cells are almost empty. The nuclei are still prominent, however, except in the carpogonium (*cp.*). This has lost its trichogyne, but it will be noticed that it contains two nuclei. This suggests that the same happens here as in *Spermothamnion Turneri* (Drew, 1934, p. 562), namely, that the fertilization nucleus divides twice to give four nuclei, two of which remain in the carpogonium and one passes into each of the two sporogenous cells (*sp.c.*). These sporogenous cells in the procarp figured are in open connexion with the auxiliary cells (*a.c.*), although the nuclei of the latter have not yet been cut off in the foot cells. The sporogenous cell (*sp.c.* 1) nucleus to the right has divided and one of the daughter nuclei has already passed into the auxiliary cell. The nucleus in the other sporogenous cell (*sp. c.* 2) is in prophase and sixty-four chromosomes are present. This nucleus is figured separately and at greater magnification in Fig. 10*a*. In the figure one of the chromosomes is not shown, as it is behind the nucleolus. In the corner of the auxiliary cell farthest from the sporogenous cell, and where the foot cell forms, is the auxiliary cell nucleus, also in prophase. There are thirty-two chromosomes present, four of which do not appear in the figure as they are behind the nucleolus (Fig. 10*b*). These two nuclei provide proof of a nuclear fusion, and this is further supported by the fact that the filament bearing this procarp is haploid. A comparison of this procarpic branch with that of *Spermothamnion Turneri* (Drew, 1934, figured on Pl. 13, Fig. 40) will emphasize the similarity between the procarpic branches of these two species. That of *S. Turneri* is slightly older and viewed from a position at right angles to that of *S. Snyderae*.

After the fusion nucleus enters the auxiliary cell the foot cell is cut off and further divisions take place to form the gonimoblasts. The carpospores are formed from the outermost cells of the gonimoblasts and are uninucleate.

Unlike *S. Turneri*, the cystocarps have no enveloping filaments (Text-fig. 11).

Abnormalities. Although there is usually only one cell above the fertile segment of the procarp, a second is by no means uncommon, as in the case of the procarp of Fig. 10. This does not appear to hinder the further development of the procarp, and in only one case has any division of this second segment cell been seen. Two pericentral cells have been formed from this extra segment, and the fertile segment below has not only completed its divisions but bears a mature carpogonial branch.

The carpogonial branch not infrequently degenerates before fertilization or divides irregularly to form a mass of cells. In the case of one such procarp, two binucleate and one uninucleate sporangium-like cells had arisen from the mass.

On two occasions procarps with two carpogonial branches have been seen. The second branch appears to have developed from the second auxiliary mother-cell in one example, but it has not been possible to ascertain the place of origin of the second carpogonial branch of the other procarp. A similar occurrence has been reported by Westbrook (1930) for *Compsothamnion thuyoides*.

Abnormal numbers of chromosomes have been noted already in the sporangia, and a similar abnormality has been found in a procarp. The procarp in question has two cells above the fertile segment, and whereas the nucleus of the lower has thirty-two chromosomes, that of the first pericentral has in the neighbourhood of sixty-four. The whole branch shows signs of degeneration and is abnormal. This possibly explains the condition of the second nucleus, which is the abnormal one, as the plant on which the procarp is borne is haploid.

V. Discussion

This investigation has shown that *Spermothamnion Snyderae* has diploid sporangia-bearing plants and haploid sexual plants and is a diplobiontic alga. In broad outline, therefore, the life-history of *S. Snyderae* is like that of many of the other Florideae, but the interest of the plant lies in the type of sporangium. The sporangium initial is multinucleate, and as the evidence available suggests that all the nuclei divide twice (as in a tetrasporangium), the mature sporangium contains a large number of uninucleate spores, normally a multiple of four. The first division in the sporangium is meiotic, and the only difference between this polysporangium and a tetrasporangium lies in the number of spores formed. The polysporangium of *S. Snyderae* must therefore be considered as a compound tetrasporangium, the two being undoubtedly homologous and the one possibly a development of the other.

The discussion centres round the sporangium and falls into three parts: the first a comparison with other Florideae with multinucleate sporangium initials, the second a consideration of species with sporangia other than tetrasporangia, and the third a consideration of the origin of multinucleate sporangium initials.

Other red algae known to have multinucleate sporangium initials as a normal event are Martensia (Svedelius, 1908) and *Nitophyllium punctatum* (Svedelius, 1914). In the case of the former, the material was not in sufficiently good condition to follow the finer details, but it appeared to behave in exactly the same way as the subsequently investigated *N. punctatum*. Although the sporangium initial of this alga is multinucleate when formed, the number of nuclei is further increased by mitotic divisions up to twelve or more. Then most of these daughter nuclei degenerate, but those remaining enter the prophase of the heterotype division. Only one completes the process, however, and as a result of a second division, four haploid nuclei result and a normal tetrasporangium is formed. The differences in the development of this sporangium and that of *S. Snyderae* are sufficiently obvious to make further remarks unnecessary. It is of interest to note, however, that Svedelius cites an abnormality in *N. punctatum*, which, he says, suggests that other nuclei besides the dominant one are facultative tetrasporangium mother-nuclei. He questions why only one develops and considers it theoretically possible for them all to survive. *S. Snyderae* is an alga, where this is actually the case and even the normal state of affairs. A preliminary investigation of a species of Pleonosporium from the Pacific, carried out some years ago, suggested that its polysporangium is like that of *S. Snyderae*, further supporting Svedelius's proposition.

There are Florideae, especially in the Ceramiales, which have sporangia, other than tetrasporangia. Without cytological examination of these sporangia a detailed classification is unsafe, but it seems likely that they fall into two groups:

(*a*) Those which may not be homologous with tetrasporangia and are purely accessory, possibly more in the nature of gemmae, e.g. the parasporangia of *Plumaria elegans* (Bonnem.) Schmitz. These will not be discussed further now as they are under investigation.

(*b*) Those which are homologous with tetrasporangia, e.g. the polysporangia of *S. Snyderae*. Polysporangia are known for several species, a fairly comprehensive list of which is given by Westbrook (1930). It remains to be discovered how many of these polysporangia are the result of multinucleate initials. Westbrook (1930) suggests that the octosporangia of *Compsothamnion thuyoides* (Smith) Schmitz originate from binucleate initials, and this writer made the same suggestion in the case of *Spermothamnion Turneri* (1934). In opposition to these species is *Gonimophyllum Skottsbergii* Setchell (Kylin, 1924). The sporangium initial in this species is uninucleate but thirty to fifty spores are formed. No evidence of when the meiotic division takes place is given by Kylin when describing the species.

The species in Westbrook's list can be divided into two groups: those which have polysporangia only, as *S. Snyderae*, and those which have tetrasporangia as well as polysporangia, the former usually predominating. Species such as the latter would appear to be intermediate between those with tetrasporangia only and those with polysporangia only. In addition, this group emphasizes the homology of the two sporangia and suggests that one has originated from the other. In the case of filamentous species with multinucleate vegetative cells, the presence of several nuclei in the sporangium initial becomes the expected rather than the unexpected condition, as the initial is merely a transformed portion of an apical cell or a lateral outgrowth, which would normally be uninucleate. The formation of a uninucleate initial involves either considerable rearrangement or degeneration of the nuclei.

On the assumption that the number of nuclei present in the sporangium initial is bound up with the number of nuclei in the somatic cells of the plant, the question whether polysporangia or tetrasporangia appeared first in the course of evolution will only be answered when it is known whether uninucleate or multinucleate vegetative cells are the more primitive. While there seems much to be said for considering them as parallel developments in the Florideae, it should be noted that the simplest forms reproduce by uninucleate monospores.

At present there seems to be no reason for comparing a polysporangium to a unilocular sporangium of the Phaeophyceae.

VI. Summary

1. *Spermothamnion Snyderae* Farlow reproduces sexually in the same way as other Ceramiaceae, but asexually only by polyspores.

2. The polysporangia are borne on diploid plants. The sporangium mother-cell may contain from two to nine nuclei, all of which undergo a reduction division. This takes place in precisely the same way as in the tetrasporangium of *Spermothamnion Turneri* (Mert.) Aresch. The haploid number of chromosomes in the nuclei of the developing sporangium is thirty-two.

3. There is no direct evidence available of the number of nuclear divisions in the sporangium, but a comparison of the number of nuclei present at diakinesis with the number of mature uninucleate spores indicates that there are two. The presence of maturing sporangia with twelve, twenty, and twenty-eight nuclei confirms this.

4. The polysporangium of *S. Snyderae* may therefore be considered as homologous with the tetrasporangium of other Florideae, the only difference being that instead of four spores there is a multiple of four.

5. The sexual plants, which are dioecious, are haploid. The spermatangial branches are like those already described for the genus, and the procarps resemble those described for *S. Turneri*. The cystocarps develop in the same way as those of the latter species but have no enveloping filaments.

6. The life-history of *S. Snyderae*, therefore, is like that of a typical diplobiontic alga.

7. Various abnormalities both of structure and in the chromosome complement are recorded.

The material for this research was collected during a stay in the United States, made possible by the Commonwealth Fund of New York. The writer wishes to express her thanks to the Founders of that Fund, to Professor W. H. Lang for his continued interest, and to the Council of the University of Manchester for laboratory facilities.

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EXPLANATION OF PLATE XXI

Illustrating Kathleen M. Drew's paper on '*Spermothamnion Snyderae* Farlow; a Floridean Alga bearing Polysporangia'.

Fig. 1. Cell about to divide into sporangium initial and stalk cell. Note apical portion destined to become sporangium contains three nuclei, already slightly larger than those in basal portion of cell. × 520.

Fig. 2. Young sporangium containing four nuclei in synizesis. × 520.

Fig. 3. Older sporangium, the three nuclei (*a*, *b*, *c*) having reached late diakinesis. × 520.

Fig. 3*a*. Nucleus *a* of Fig. 3. Gemini number thirty-two. One partly under nucleolus, another completely. Structure marked *p* probably extra-nuclear. × 2,120.

Fig. 3*b*. Nucleus *b* of Fig. 3. Of the thirty-two gemini present one is behind the nucleolus. × 2,120.

Fig. 3*c*. Nucleus *c* of Fig. 3. Thirty-two gemini present, four behind nucleolus. Structures marked *p* probably extra-nuclear. × 2,120.

Fig. 4. Nucleus *a* of sporangium of Text-fig. 9. × 2,120.

Fig. 5. Nucleus of sporangium in the prophase of homotype division. Haploid number of chromosomes, some of which occur in pairs. Large body *p* probably not a chromosome. × 2,120.

Fig. 6. Apical portion of branchlet of male plant, showing first stages in development of spermatangial branchlets (*sp. br.*). × 520.

Fig. 6*a*. Nucleus marked *c* in oldest spermatangial branch (*sp. br.* 3) of Fig. 6. Haploid number of chromosomes visible and one doubtful chromosome behind nucleolus. × 2,120.

Fig. 7. Apical cell of branch just before division into upper uninucleate cell from which procarp will develop and lower multinucleate cell. Note difference in size of nuclei. × 970.

Fig. 8. Very early stage in procarp development. Lower of two uninucleate cells is fertile segment and has nucleus in prophase. × 970.

Fig. 8*a*. Nucleus of fertile segment of Fig. 8. Thirty-two chromosomes present. × 2,120.

Fig. 9. Much later stage in procarp development. Carpogonial branch three-celled (*cp. br.*). Lower two cells binucleate, the apical with a single nucleus in prophase. Third pericentral cell left blank to show outline of second pericentral (= supporting-cell) and sterile cell (*st. c.*) on opposite side of procarp, as well as details of carpogonial branch. × 970.

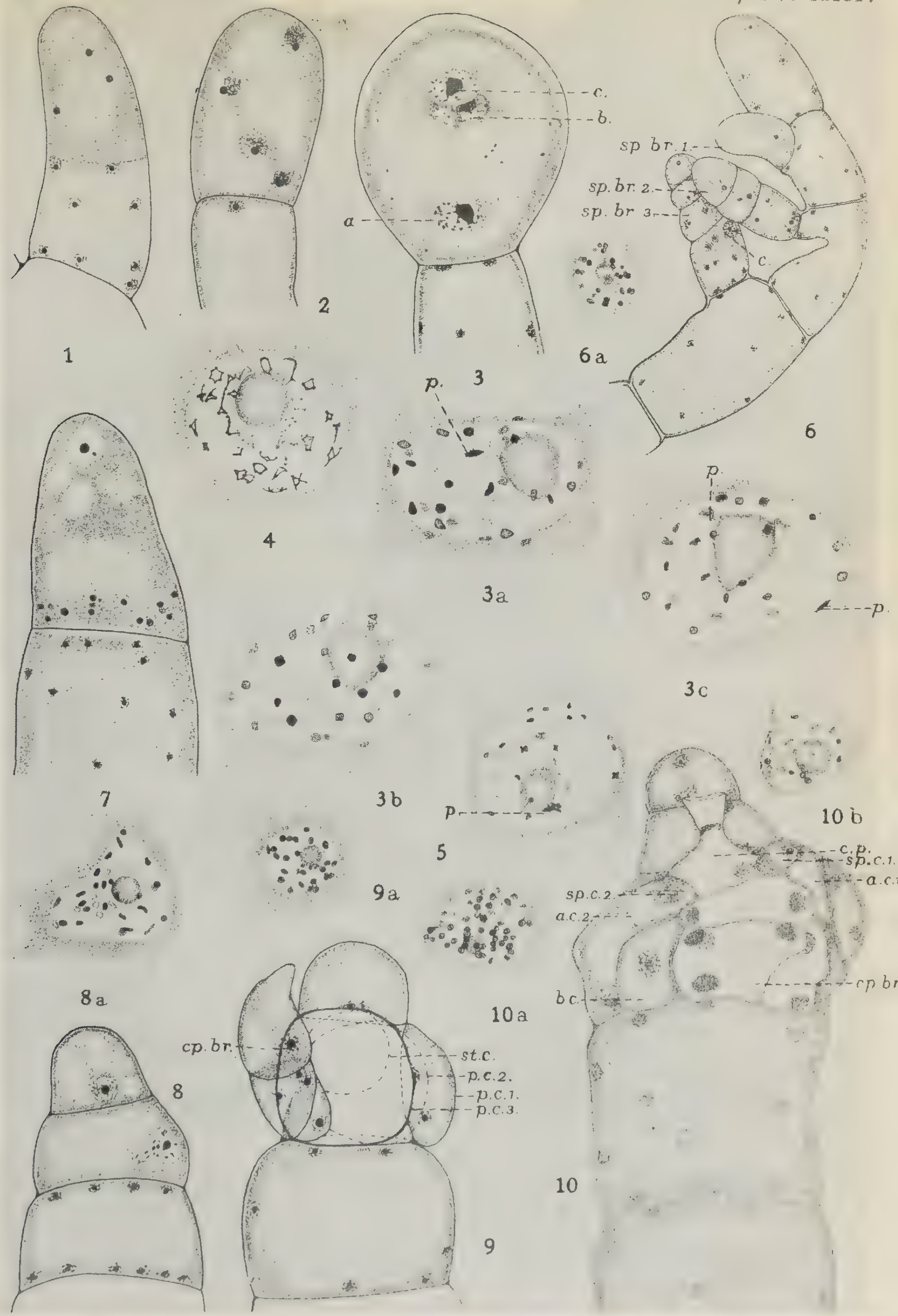
Fig. 9*a*. Nucleus of apical cell of carpogonial branch of Fig. 9. Thirty-one chromosomes distinguishable. × 2,120.

Fig. 10. Procarp showing changes following on fertilization. Cells on back side not indicated. Cells of carpogonial branch (*cp. br.*) contain very little protoplasm. Carpogonium (*cp.*) has lost trichogyne and contains two degenerating nuclei. Sporogenous cells (*sp. c.*) in open connexion with auxiliary cells (*a. c.*). Nuclei of sporogenous cell and auxiliary cell to left in prophase. × 970.

Fig. 10*a*. Nucleus of sporogenous cell (*sp. c.* 2) of Fig. 10. Sixty-four chromosomes present but one behind nucleolus. × 2,120.

Fig. 10*b*. Nucleus of auxiliary cell (*a. c.* 2) of Fig. 10. Haploid, but four chromosomes hidden by nucleolus. × 2,120.

Note: In multinucleate somatic cells the nuclei on the upper side only of the cell are shown.



K. M. D. del. Huth, Stubbs X. Kent.

DREW — SPERMOTHAMNION SNYDERAE.

Observations on the Oxygen Uptake of Isolated Plant Tissue

I. The Effect of Phosphate and of Added Carbohydrate

BY

JOHN CALDWELL

AND

JANE MEIKLEJOHN

(Departments of Plant Pathology and General Microbiology, Rothamsted Experimental Station, Harpenden, Herts.)

With one Figure in the Text.

INTRODUCTION

IN the course of an examination of the possibility of oxygen uptake by the agent of aucuba virus disease of tomato, a number of studies of the behaviour of plant tissue in Barcroft respirometers were made. The experiments with the virus material yielded negative results; in no instance, with crude virus juice filtered through Pasteur-Chamberland L3 candles, or with purified and concentrated virus material (Caldwell, 1935), was there any indication that oxygen was taken up, even when large quantities of active virus-agent were present in the experimental material.

Subsequent experiments, performed with tissue of healthy and diseased plants, while they threw little light on the problem of virus disease, yet gave results which are suggestive of other lines of investigation, and some of these results are recorded in this paper.

MATERIALS AND METHODS

The material used was slices of tomato stem tissue prepared by removing, under sterile conditions, the extra-cambial tissues and then cutting the remaining tissue longitudinally into slices about 2·5 cm. long by 0·5 mm. thick. In preliminary experiments it was found that, if thicker blocks of tissue were used, the oxygen uptake was limited by the rate of diffusion into the block; the type of results obtained are illustrated in Table I.

As filling the Barcroft bottles with oxygen instead of air (to increase the external oxygen concentration) caused no measurable increase in the oxygen uptake of slices 0·5 mm. thick, it was concluded that the slices were sufficiently thin to allow of adequate diffusion of oxygen into the interior of the tissue (cf. Warburg, 1930).

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The apparatus used for the measurement of oxygen uptake consisted of Barcroft differential manometers; the customary technique was employed (Dixon, 1934). All the measurements were made at 26° C. The oxygen uptake is expressed throughout as μl. of gas (dry at N.T.P.) taken up per hour, per gramme fresh weight of tissue.

TABLE I

Comparison of Blocks and Slices

Method of cutting.	Tissue suspended in.	Oxygen uptake (μl./gm./hr.)		
		(1)	(2)	(3)
Whole block . .	M/10 KH_2PO_4	64	56	61
Half blocks . .	”	73	74	78
Slices . . .	”	81	78	84
Whole block . .	M/30 KH_2PO_4	—	63	—
	M/30 KH_2PO_4+0·5 per cent. glucose	—	70	—
Half blocks . .	M/30 KH_2PO_4	—	62	—
	M/30 KH_2PO_4+0·5 per cent. glucose	—	79	—
Slices . . .	M/30 KH_2PO_4	—	70	—
	M/30 KH_2PO_4+0·5 per cent. glucose	—	88	—

The procedure in a typical experiment was as follows: the slices of tomato stem, prepared as described above, were divided into six approximately equal batches. Each batch (weighing between 0·1 gm. and 0·7 gm.) was placed in a tared Barcroft bottle containing 3 ml. of sterile dilute potassium dihydrogen phosphate (KH_2PO_4) solution, and the whole weighed. The usual roll of filter-paper and KOH solution were added, the apparatus placed in the bath and brought to equilibrium, and readings were then taken at intervals over a period of six hours. Dilute acid potassium phosphate was used in which to suspend the slices as it is a non-poisonous medium of an acidity comparable with that of tomato juice. In the earlier experiments the concentration of phosphate was varied; in the later experiments, only one concentration of phosphate (M/30) was used, but sugar was added to some of the bottles, the required amount being dissolved beforehand in the phosphate medium.

In all the work recorded in this paper precautions were taken to ensure that no bacterial contamination took place; when on one occasion this did occur the increase in oxygen uptake late in the six-hour period was so marked that it was easy to see that something unusual had happened.

Note on the accuracy of the results.

Every figure for oxygen uptake given in the tables is a mean value, derived from six samples in Tables II and III, and two or three samples in the later tables. The actual results from which the means are taken varied over a fairly wide range. The main factor contributing to this variation was probably the difficulty of obtaining uniform sets of samples of the relatively large size necessitated by the small oxygen uptake of plant tissue. Where the mean oxygen uptake was above 40 μl./gm./hr., the average standard deviation was found to be about 10 per cent. of the mean. It follows that differences, due to treatment, of less than 10 per cent. of the mean value of the control cannot be detected. Most of the differences reported are of a higher order; significance tests were applied to them all, and those differences which were found not to be significant are so marked in the tables.

RESULTS

1. *Phosphate concentration and age of tissue.*

In the first series of experiments tissue from healthy plants was used; the plants were of two different sizes; plants in the 5th leaf stage (referred to as 'very young' plants), and plants in the 6th to 12th leaf stage (referred to as 'young' plants). The oxygen uptake of tissue from plants of both types was measured in distilled water, and in different concentrations of phosphate. The results are given in Table II.

TABLE II

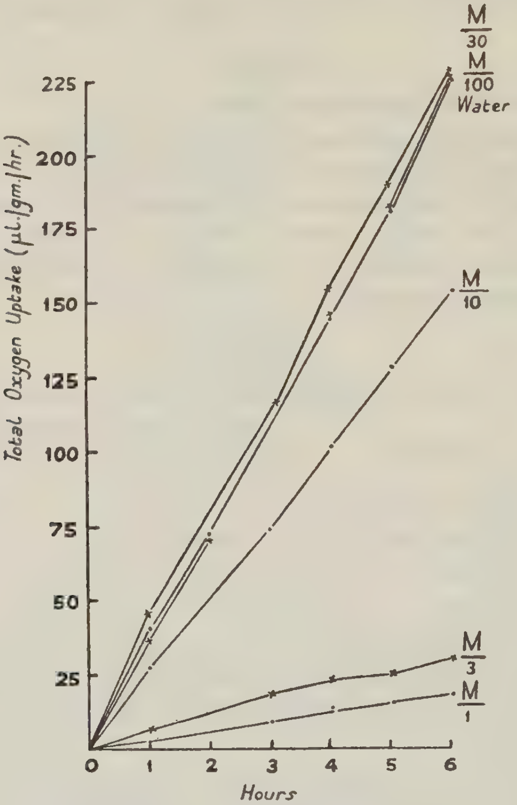
Oxygen Uptake of Slices of Tomato Stem (Healthy Plants)

	Mean oxygen uptake (μl./gm./hr.).	
Concentration of KH_2PO_4.	Very young plants.	Young plants.
M	8·1	—
M/3	14·0	39·5
M/10	59·5	114·2
M/30	87·8	137·4
M/100	86·0	112·3
Water	85·9	114·5

It will be observed that the level of oxygen uptake is consistently higher in the tissue derived from young, as compared with very young, plants, and also that the oxygen uptake is depressed in concentrations of phosphate stronger than M/30.

[An examination of the data from which the mean values were derived showed that (*a*) the mean value for oxygen uptake at any concentration of phosphate from M to M/30 was significantly different from the means at adjacent concentrations, and (*b*) that the value given by tissue from very young plants at any concentration of phosphate was significantly different from the value given by tissue from young plants at the same concentration.]

In the diagram are given progress curves for the oxygen uptake of individual samples of about equal weight, one from each experiment of the first series; it will be seen that the depressing action of the stronger solutions of phosphate is apparent throughout the period of observation. It will also be observed that the rate of oxygen uptake is very nearly constant in every case (i.e. the 'progress curves' approach very closely to straight lines), and is maintained at the same level right to the end of the six-hour period. This constant rate of oxygen uptake appears to be characteristic of the stem tissue, as it was observed in every sample throughout the experiments. Marked curling of the stem slices was observed to take place in the stronger solutions of phosphate (M to M/10); but the slices never became flaccid in the six-hour period, and indeed retained their normal turgidity even after standing for twenty-four hours.



Very young plants. Oxygen uptake of individual samples of tissue in water and in various concentrations of acid potassium phosphate.

The first series of experiments was then repeated exactly with plants infected with aucuba mosaic virus, and in addition a few experiments were performed with tissue from old diseased plants (i.e. plants that had flowered.)

It will be seen that the results agree with those obtained with healthy plants, both in the depression of oxygen uptake in the stronger concentrations of phosphate, and in the consistently lower level of respiration in tissue from plants in the 5th leaf stage, as compared with slightly older plants.

As might be expected, the tissue from old plants has a still lower level of respiration (cf. Kidd, West, and Briggs, 1921). But the low level of oxygen uptake in tissue from very young plants, observed with both healthy and diseased plants, is a more unexpected result.

That a low respiration rate is not an intrinsic property of young tissue is shown by a consideration of the results obtained when samples taken from the apex of the stem of a plant in the 6th to 12th leaf stage are compared with samples taken farther and farther down the stem. A plant in this stage is still actively growing, and the apex of the stem is composed of more recently formed, and therefore younger, tissue than the lower part of the stem.

TABLE III

Oxygen Uptake of Slices of Tomato Stem (Diseased Plants)

Concentration of KH_2PO_4.	Mean oxygen uptake (μl./gm./hr.). Very young plants.	Young plants.	Old plants.
M	13·9	—	14·3
M/3	27·2	55·7	35·0
M/10	57·7	102·7	54·3
M/30	78·0	121·1	47·8
M/100	*100·5	109·0	—
Water	*88·1	106·7	—

* (2 samples only.)

TABLE IV

Position Gradient of Respiration

Position on stem.	No. of samples.	Mean oxygen uptake (μl./gm./hr.).
Apex . . .	5	163
2nd internode .	6	148
Middle part . .	3	97
,, . .	3	124
,, . .	3	114
,, . .	3	104
Near ground-level.	3	98

The results of one such experiment are given in Table IV; a single well-grown healthy young plant was used, and the oxygen uptake of samples taken farther and farther down the stem measured in M/30 KH_2PO_4.

In a similar experiment, with a virus plant of the same age, samples of tissue from the apex of the stem had a mean oxygen uptake of 169 μl./gm./hr., while the main part of the stem gave mean values of 100–120 μl. So in both cases the youngest tissue, taken from the apex of the stem, had a much higher respiration rate than the slightly older tissue from the lower part of the plant.

It is therefore unlikely that the low level of oxygen uptake in tissue from plants in the 5th leaf stage was caused by the youth *per se* of the tissue, and another cause must be looked for. Among the probable causes is a lack of available respiratory substrate; to obtain further information on this point the experiments reported below in section 2 of this paper were carried out.

2. *The effect of added carbohydrates.*

A series of experiments was carried out to test the effect of adding carbohydrate to tissue from plants of different ages. The carbohydrates used were the monosaccharides glucose and fructose; their effect on the oxygen uptake of tomato tissue appeared to be identical (except in one anomalous experiment which we were unable to confirm).

TABLE V

Addition of Glucose to Tissue from Old Plants

Type of plant.	Glucose per cent.	Mean oxygen uptake (μl./gm./hr.). Control.	Treated.	Increase per cent.
After flowering .	0·5	95	99	5 (not sig.)
,, .	0·5	95	95	0
Very old . .	0·5	48	56	15 (not sig.)

TABLE VI

Addition of Carbohydrate—Young Plants (Healthy)

Carbohydrate.	Concentration per cent.	Mean oxygen uptake (μl./gm./hr.). Control.	Treated.
{Glucose . .	0·2	*96	96
{Fructose	0·2	—	98
Glucose . .	0·2	133	135
,, . .	1·0	143	153
Fructose . .	1·0	163	161
Glucose . .	0·5	103	105 (plant 132 hours in dark).

* (2 samples).

[M/30 acid phosphate was used as the suspension medium, as it had already been found to give the maximum oxygen uptake; in each experiment a sugar solution of the required strength (0·2, 0·5, or 1·0 per cent.) was made up in sterile phosphate and added to three of the Barcroft bottles, while phosphate alone was added to the other three, thus providing a separate control for each experiment. (If glucose and fructose were used in the same experiment, they were added to two bottles each, leaving two control samples.)]

In the first set of experiments old plants were used; only a limited amount of material, and that all from virus plants, was available. The results, given in Table V, indicate that a negligible increase of oxygen uptake was obtained on the addition of glucose, although the uptake of the control samples was small.

When 'young' plants (in the 6th–12th leaf stage) were used, the level of oxygen uptake in the controls was rather higher; but in this case also there was not a significant increase in oxygen uptake on the addition of carbohydrate. Some typical results are given in Table VI, which includes two experiments with a 1 per cent. concentration of sugar, and one where the plant was kept in the dark before the experiment.

When tissue from very young plants (5th leaf stage) was used, on the other hand, a very marked increase in oxygen uptake was produced in every case by the addition of carbohydrate. The results of some experiments with healthy 5th leaf-stage plants are given in Table VII. In the later experiments of this set the plant was kept in the dark for some time before the experiment;

TABLE VII

Very Young Healthy Plants—Addition of Carbohydrate

Hours in dark.	Carbohydrate.	Concentration per cent.	Oxygen uptake (μl./gm./hr.). Control.	Treated.	Increase per cent.
0	Glucose	0·2	*84	107	27
	Fructose	0·2	—	107	27
0	Glucose	0·2	60	105	75
0	Glucose	0·2	68	124	82
16	Glucose	0·2	*46	65	46
	Fructose	0·2	—	67	50
40	Glucose	0·5	*47	79	58
	Fructose	0·5	—	76	59
48	Glucose	0·5	46	88	91
64	Glucose	0·5	*37	84	127
	Fructose	0·5	—	67	80
72	Glucose	0·5	41	59	44

* (2 samples).

TABLE VIII

Very Young Diseased Plants—Addition of Glucose

Hours in dark.	Concentration per cent.	Oxygen uptake (μl./gm./hr.). Control.	Treated.	Increase per cent.
16	0·5	19	51	170
96	0·5	34	83	144
200	0·5	24	46	100

this treatment reduced the oxygen uptake of the controls, and augmented slightly the effect of added carbohydrate. The increase in oxygen uptake is expressed as a percentage of the control uptake; i.e. by the expression $\frac{(b-a)\,100}{a}$, where a is the mean oxygen uptake of the control, and b of the treated, tissue.

Owing to a shortage of material only a few experiments could be performed on diseased 5th leaf-stage plants; the results are given in Table VIII.

These three experiments, as far as they go, show that the oxygen uptake of the control tissue is reduced to a greater extent in diseased than in healthy plants, by keeping the plant in the dark before the experiment, and that the addition of glucose produces a proportionately greater rise in oxygen uptake.

Respiratory quotient.

Some measurements of the respiratory quotient were performed on tissue from healthy young plants. The technique used was that described by Dixon

(1934), in which the measurements of oxygen uptake, carbon-dioxide output, and bound carbon dioxide are performed on three different samples; the estimate of the respiratory quotient is therefore only approximate.

TABLE IX

Respiratory Quotient

Expt. no.	Oxygen uptake (μl./gm./hr.).	R.Q. (CO_2/O_2).
1	137	0·94
2	184	1·02
3	158	1·03
4	158	1·02
5	144	1·01

None of the figures obtained for the respiratory quotient was significantly different from unity.

DISCUSSION

The method employed in this work appears to give, with a very small amount of material, an adequate measurement of the respiration of plant tissue. That the measurements of oxygen uptake recorded were in fact due to normal aerobic respiration is indicated by the following observations: (*a*) the slices of stem tissue were invariably found to take up oxygen at a constant rate which was maintained over the whole period of an experiment; (*b*) the estimates obtained of the respiratory quotient approached closely to unity; (*c*) the tissue slices used showed no sign of flaccidity after standing for twenty-four hours.

The effect of phosphate.

As the oxygen uptake of slices suspended in distilled water was only slightly less than that of slices suspended in M/30 phosphate, it is evident that there was no question of phosphate starvation in the tissue used. M/30 phosphate was probably effective in producing the largest oxygen uptake observed because it approached most nearly, in acidity and tonicity, to the cell-sap of all the solutions used. It certainly caused less curling of the tissue slices than the other solutions. The marked depression of respiration observed in the presence of M/10 or stronger concentrations of phosphate is probably due to their being hypertonic to the cell-sap; it is interesting to compare with our results those of van Heyningen (1935) on the oxygen uptake of a variety of animal tissues. Van Heyningen criticizes the work of Dixon and Elliott (1929) on the ground that they used a buffer solution containing M/10 phosphate in their experiments, and that this concentration was too strong; the medium that he used contained M/40 phosphate, and in this medium he obtained consistently higher values of oxygen uptake than those recorded by Dixon and Elliott for the same animal material.

The effect of age on oxygen uptake.

In any study on the effect of age on the respiration of isolated parts of a plant it is important to distinguish between the age of the plant from which the part has been removed and the stage of development reached by that particular part. That a failure to make this distinction may lead to misconceptions of the effect of age on physiological processes has been shown by Richards, who points out (1934) the fallacies underlying the method of estimating the effect of age on, e.g. respiration, by performing measurements on successive leaves taken from the same plant, and states that corresponding leaves from plants of different ages must be used in such an estimation, for, unless this is done 'it is impossible to separate effects which may be possibly ascribed to age as such from those due to change in conditions of nutrition, etc.' This point is emphasized in the work reported in this paper; for instance, the extreme top of a plant in the 6th to 12th leaf stage may be regarded as young tissue in one sense, as it is the last formed part of the plant; but we have shown that it has a very much higher rate of respiration than tissue removed from a very young plant. The tissues at the top of a mature plant are not physiologically equivalent to those of a very young plant.

In a well-grown young plant there must be an ample supply, throughout the plant, of respirable material formed by photosynthesis, and under these circumstances very young tissue exhibits a higher rate of oxygen uptake than older tissue. It is therefore probable that the low rate of oxygen uptake exhibited by tissue from very young plants is caused by a lack of available substrate, most of the respirable material having been used up in tissue formation, and not by a lack of activity in the respiratory enzyme system.

This suggestion is confirmed by the results obtained on the addition of sugar to the respiratory tissue. In young plants, where both an efficient respiratory system and an ample supply of respiratory substrate are present, the oxygen uptake of untreated tissue is high and is not raised by the addition of carbohydrate. In both very young and very old plants the oxygen uptake of untreated tissue is low, but from different causes. In old plants the uptake is only very slightly raised by added carbohydrate, and the low respiration rate is presumably due to a decline in the activity of the respiratory system. In very young plants the respiratory system is highly active, but there is a lack of available substrate, and accordingly the addition of glucose or fructose produces a very marked rise in oxygen uptake.

The results obtained with added carbohydrate also indicate the one point of difference between healthy- and virus-diseased tissue that was observed in these experiments. It appears that respiratory substrate is lost to a greater extent from virus plants when they are kept in the dark than from healthy plants. Glucose, added to the tissue from starved virus plants, produced a greater proportional increase of respiration than in the corresponding normal tissue. The incompleteness of these results prevents any very definite conclusion being drawn from them, but they partly confirm a suggestion made by Caldwell (1934) that the enzymes concerned in the preliminary stages of respiration were more active in virus-diseased than in healthy plants. The preliminary experiments, with extracted and with purified juice from virus plants, gave no indication that the virus agent itself had any oxygen uptake. This confirmed the view that the increase in the respiration rate of the tissues of diseased plants was not associated with the respiration of the virus agent itself.

SUMMARY

1. The oxygen uptake of thin slices of tomato stem tissue was measured in Barcroft respirometers, and found to be maintained at a constant rate over a six-hour period.

2. The highest values for oxygen uptake were observed in presence of M/30 potassium dihydrogen phosphate; measurements in distilled water gave slightly lower values, and stronger solutions of phosphate produced a marked depression of oxygen uptake.

3. Tissue from very young plants, in the 5th leaf stage, showed a lower level of oxygen uptake than tissue from slightly older plants, up to the 12th leaf stage. A low level of oxygen uptake was also observed in tissue from old plants that had flowered.

4. The small oxygen uptake of tissue from very young plants was markedly raised by the addition of glucose or fructose, but no such rise was observed on adding sugar to tissue from very old plants.

5. It is concluded that the oxygen uptake is limited in old plants by the activity of the respiratory enzyme system, and in very young plants by the amount of available respiratory substrate.

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Observations on the Oxygen Uptake of Isolated Plant Tissue

II. The Effect of Inhibitors

BY

JOHN CALDWELL

AND

JANE MEIKLEJOHN

(*Departments of Plant Pathology and General Microbiology, Rothamsted Experimental Station, Harpenden*)

With three Figures in the Text.

INTRODUCTION

DURING an attempt to study the possible effects of aucuba mosaic virus upon the respiratory activity of infected plants a number of observations were made on the oxygen uptake of excised tissue from the stem of the tomato (*Lycopersicum esculentum*). Some of these observations have been reported in a previous paper (Caldwell and Meiklejohn, 1937), which deals with the effect of phosphate and of added carbohydrate. In the present paper are described a further series of experiments, in which substances known or thought to inhibit enzyme action were added to the tomato tissue.

METHODS

The methods and material used have been described in detail in the earlier paper. The stem tissue was cut into slices 0·5 mm. thick, which were suspended in 3 ml. of M/30 acid potassium phosphate (KH_2PO_4), and their oxygen uptake at 26° C. was measured by means of Barcroft differential manometers over a six-hour period. The method, previously described, of providing a separate control for each experiment, was followed throughout; the inhibitor was added to three of each set of six Barcroft bottles after weighing, and just before they were attached to the manometers. These bottles contained either 2·9 or 2·7 ml. of phosphate solution, and either 0·1 or 0·3 ml. of a solution of the poison of appropriate strength was added to bring the volume up to 3 ml. (e.g. to obtain a concentration of M/300 KCN in the bottles, 0·1 ml. of an M/10 solution of potassium cyanide was added to 2·9 ml. of phosphate solution). The amount of poison added is expressed throughout in terms of the concentration in the bottles at the beginning of the experiment.

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The amount of oxygen taken up is expressed as μl. of gas (dry at N.T.P.) per hour per gramme fresh weight of tissue, and each figure given in the tables is the mean of three parallel samples. The degree of inhibition of oxygen uptake, where this occurred, is expressed as a percentage of the uptake of the control, i.e. by the expression $\frac{(a-b)}{a}$ 100, where a is the mean oxygen uptake of the control, and b of the treated material. The reality of the observed differences between the control and the treated material was tested by calculating the standard errors of these mean values (Fisher, 1934). As was stated in the previous paper, the variation of the individual readings with this material is such that differences of the order of 10 per cent. of the control cannot be detected. In a few cases small differences between the mean values for control and treated material have been found not to be significant, and are marked accordingly in the tables. The differences not so marked have been tested and found to be highly significant.

RESULTS

(a) *Cyanide.*

The addition of potassium cyanide to the phosphate solution in which the stem slices were placed produced a very marked inhibition of oxygen uptake. As will be seen by reference to Table I, an inhibition amounting to 85 per cent. of the uptake of untreated material was produced by M/300 cyanide; on raising the concentration of cyanide to M/30 no further inhibition was produced, so that the cyanide-stable part of the respiratory system accounts for about 15 per cent. of the total oxygen uptake. These figures are very similar to those obtained by van Heyningen (1935) with animal tissue, both in the amount of cyanide required for maximal inhibition, and in the degree of inhibition (80–90 per cent. for most animal organs) produced.

TABLE I

Effect of Cyanide on the Oxygen Uptake of Tomato Tissue

Concentration of cyanide.	Solution in.	Type of plant.	Oxygen uptake (μl./gm./hr.). Control.	Treated.	Inhibition per cent.
M/30	M/30 KH_2PO_4	Healthy young	127	19	85
M/300	"	"	97	21	79
"	"	"	124	20	84
"	"	Virus young	77	15	80
"	Water	Healthy young	98	14	85
"	M/30 K_2HPO_4	"	85	12	85
M/3,000	M/30 KH_2PO_4	"	114	46	61
M/30,000	"	"	104	97	5 (not sig.)
"	M/30 K_2HPO_4	"	76	85	0
"	"	"	74	72	0

It will be seen that the inhibition produced by M/300 cyanide is of the same order with virus material as with healthy, and is the same in water or alkaline phosphate as in the acid phosphate solution used as a standard. The disadvantage of using an acid solution for these experiments, however, is that there is a loss of cyanide from the solution during the experiment, which affects the results with low concentrations of cyanide. (A method for avoiding this loss has been described by Krebs (1935), but it was not employed in our experiments.) This point is brought out in Fig. 1, which represents measurements of total oxygen uptake during the course of an ex-experiment, from single samples of tissue of approximately equal weights. It will be noticed that in presence of M/3,000 cyanide, the oxygen uptake increased during the experiment; the effect of the cyanide wore off, as more and more of it was lost from the solution. In the case of M/30,000 cyanide there was an initial inhibition only; but with M/300 or M/30 there was so much cyanide present that the loss by evaporation was not noticeable.

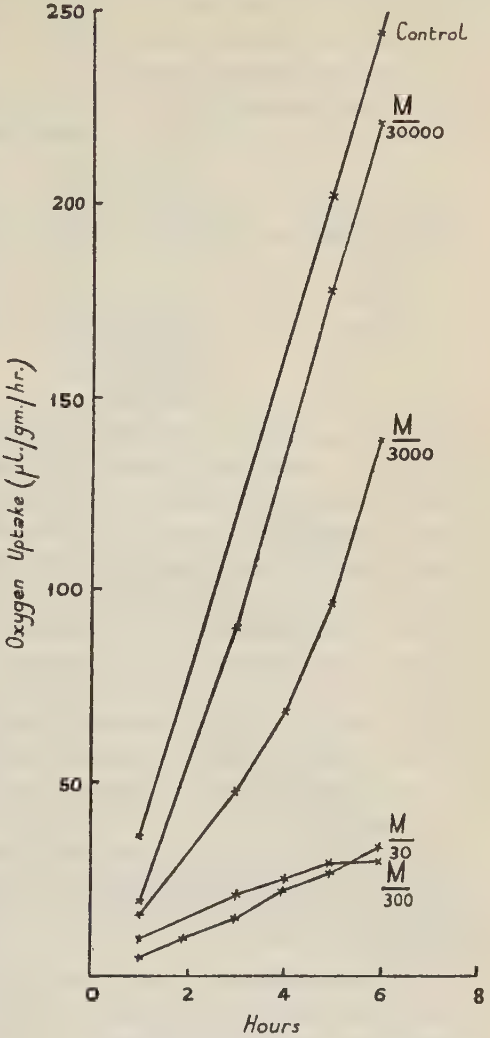

Fig. 1. Oxygen uptake of single samples of tissue in the presence of KCN.

This diagram also illustrates the fact, to which reference has previously been made, that the oxygen uptake of the control tissue was constant throughout the period of the experiment (i.e. successive readings give a straight line when plotted as a graph); this has been found to hold for untreated material throughout these experiments.

The action of cyanide in lowering the oxygen uptake is at least partly reversible, i.e. the effect disappears on the removal of the cyanide, as is shown by an experiment carried out with tobacco stem tissue (no tomato tissue being available). In this experiment the average oxygen uptake of three control samples (in M/30 KH_2PO_4) was 76 μl./gm./hr. The three treated samples were allowed to respire for one hour in an M/300 solution of cyanide in M/30 phosphate, when their average oxygen uptake was 8 μl./gm./hr., giving an inhibition of 89 per cent. The treated samples were then removed, washed in two changes of sterile phosphate solution, and placed in fresh bottles with 3 ml. of phosphate solution in each. Their average oxygen uptake, measured over four and a half hours, was then 33 μl./gm./hr., or four times the uptake in presence of cyanide, a recovery of nearly half the original rate. Total recovery was not observed, probably because the washing process removed some of the soluble carbohydrate in addition to the cyanide, and because the washing process was not absolutely complete.

As very weak concentrations of cyanide have been shown to have a stimulating effect on the respiration of some plant tissues (Hanes and Barker, 1930), and as we had observed no stimulation of oxygen uptake due to cyanide in our experiments, it was thought desirable to measure the respiratory quotient of tomato tissue in the presence of very weak cyanide, to ascertain whether there was any stimulation of carbon-dioxide output. The method described by Dixon (1934) was used, in which the oxygen uptake is measured in one apparatus and the carbon-dioxide output measured in another, while a third is used for the measurement of retained carbon dioxide. As Dixon pointed out, this direct method is not adapted to accurate measurement of the respiratory quotient, as the oxygen and carbon-dioxide estimations have to be performed on different samples. The values obtained for the respiratory quotient (given in Table II) must, therefore, be regarded as approximate only, more especially as the figures given for oxygen uptake are taken from single samples, no duplicates being possible.

TABLE II

Respiratory Quotients in Presence and Absence of Cyanide

Type of plant.	Treatment.	Oxygen uptake (μl./gm./hr.).	R.Q. $(CO_2)/O_2$.
Healthy young	+M/30,000 KCN	114	1·02
,,	Control	158	1·02
,,	+M/30,000 KCN	139	1·02
	Control	144	1·01

These results, though approximate, show no perceptible alteration of the respiratory quotient in presence of weak cyanide; if any stimulation in carbon-dioxide output took place under such conditions, it must have been exceedingly small.

Two experiments were performed to test the effect of cyanide on oxygen uptake in the presence of added glucose; for this purpose tissue from very young plants was employed, as it was known to respond to the addition of glucose (Caldwell and Meiklejohn, 1937).

In both cases, although the respiration in absence of cyanide was raised on the addition of glucose, the residual respiration in presence of cyanide was the same with or without glucose. These results agree with those obtained by Genevois (1929).

(b) *Sodium fluoride.*

Sodium fluoride produced a marked inhibition of the oxygen uptake, but only in relatively high concentrations, the maximal effect being observed in the presence of M/30 fluoride; the effect in water was the same as in phosphate solution. This inhibition is not reversible; tissue subjected to treatment with M/300 fluoride for an hour showed no recovery of oxygen uptake after washing. The results are given in Table IV.

TABLE III

Effect of Cyanide in Presence of Glucose (Very Young Plants)

Type of plant.	Treatment (in M/30 KH_2PO_4).	Oxygen uptake (μl./gm./hr.). No KCN.	KCN present.	Change in uptake (per cent. of control).
Healthy	Control	41		
	+M/75 KCN		13	−68
”	0·5 per cent. Glucose	59		+44
	+M/75 KCN		11	−80
Virus	Control	34		
	+M/150 KCN		15	−56
”	0·5 per cent. Glucose	83		+144
	+M/75KCN		14	−82

TABLE IV

Effect of Sodium Fluoride on the Oxygen Uptake (Healthy Young Plants)

Concentration of NaF.	Solution in.	Oxygen uptake (μl./gm./hr.).		Inhibition per cent.
		Control.	Treated.	
M/30	M/30 KH_2PO_4	99	8	92
”	Water	122	11	91
M/300	M/30 KH_2PO_4	130	32	75
M/3,000	”	101	92	9
M/300	”	106	44 (before washing)	
	(no recovery)		37 (after washing)	

The course of respiration of individual samples of tissue in presence of different concentrations of fluoride is shown in Fig. 2. There was no diminution in effect due to loss of inhibitor during the experiment in this case; on the contrary, an increase in inhibition as the experiment proceeds may be seen in the curve for M/300 fluoride.

(c) *Iodoacetic acid.*

Iodoacetic acid strongly inhibited the oxygen uptake of tomato tissue at very weak concentrations.

The inhibition caused by iodoacetic acid is not constant throughout the period of observation; in Fig. 3 are given the actual readings from control and treated tissue samples of approximately equal weight, which show that the inhibition increases with time. This is particularly noticeable in the curve for M/10,000 iodoacetic acid. Apparently this particular inhibitor penetrates into the tissue far more slowly than, for instance, cyanide. This inhibition is not reversible; the results obtained by allowing the tissue to respire for one hour in presence of iodoacetate, washing it with sterile phosphate, and measuring the oxygen uptake in fresh phosphate, are given in Table VI.

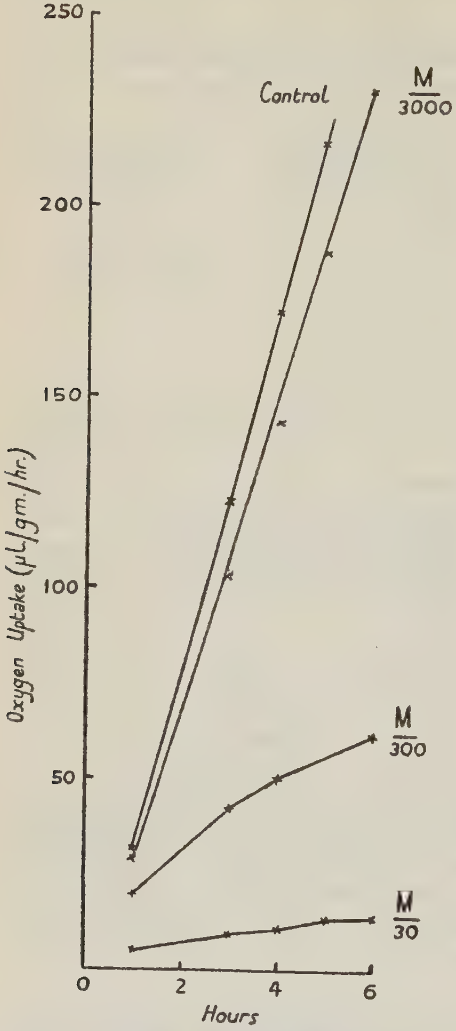

Fig. 2. Oxygen uptake of single samples of tissue in the presence of sodium fluoride.

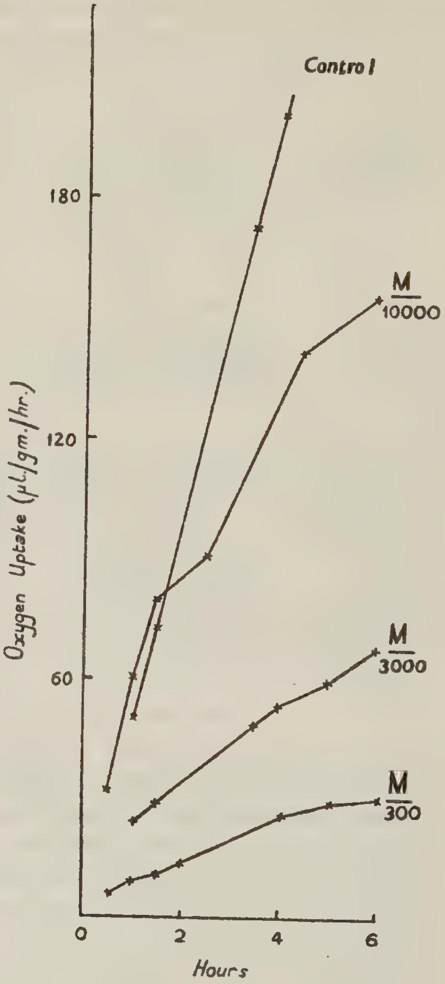

Fig. 3. Oxygen uptake of single samples of tissue in the presence of iodoacetic acid.

(The first experiment was done with tobacco stem tissue, owing to a shortage of tomato plants.) No recovery was observed after washing, even at M/10,000 iodoacetate; in fact, washing appears to reduce the oxygen uptake still further. This may be because the inhibition increases with time.

As, in some animal tissues, the oxygen uptake in presence of iodoacetate can be restored to its original value by the addition of lactate (Needham, 1932), it was thought of interest to examine the effect of iodoacetic acid on tomato stem tissue in presence of lactic acid. Two preliminary experiments with lactate alone in acid phosphate solution showed that it was only utilized to a limited extent as a respiratory substrate (first two lines of Table VII). When lactic acid was added to the phosphate solution (M/30 KH_2PO_4) without neutralization, the resulting solution had a pH value of 3·0, and this, in conjunction with iodoacetic acid, completely killed the tissue, making it flaccid, and stopping all uptake of oxygen. Another experiment was therefore performed in which the lactic acid in the acid phosphate solution was neutralized

TABLE V

The Effect of Iodoacetic Acid on the Oxygen Uptake of Tomato Tissue

Concentration of iodoacetate.	Type of plant.	Oxygen uptake (μl./gm./hr.). Control.	Treated.	Inhibition per cent.
M/300	Old healthy	83	8	91
M/3,000	"	54	12	77
"	Young healthy	132	30	77
"	"	109	33	70
M/10,000	"	141	51	64
M/30,000	Old healthy	46	31	32
M/300	Young virus	103	2	98
"	"	140	8	94
M/3,000	"	109	19	82
M/30,000	Old virus	60	53	(12) (not sig.)

TABLE VI

Effect of Washing Tissue treated with Iodoacetate (Healthy Plants)

Concentration of iodoacetic acid.	Type of plant.	Oxygen uptake (μl./gm./hr.). Control.	Treated. (before washing).	Treated. (after washing).
M/300	Old tobacco	92	30	11
M/3,000	Young tomato	122	57	24
"	"	141	26	18
M/10,000	"	141	98	72

TABLE VII

Lactic Acid and Iodoacetic Acid (Young Healthy Plants)

Concentration of iodoacetic acid.	pH.	Concentration of lactic acid	Oxygen uptake (μl./gm./hr.). Without lactic acid.	With lactic acid.
0	4·6	0·1 per cent.	67	77
0	4·6	0·5 "	40	10
M/3,000	3·0	0·5 "	38	0
"	4·8	0·5 "	28	17
"	7·2	0·5 "	*56	*60
0	7·2	0·5 "	*73	

* 2 samples.

to pH 4·8; and a third experiment was performed in a neutral buffer solution (equal parts of M/30 KH_2PO_4 and M/30 K_2HPO_4, pH 7·2).

In acid solution the lactic acid depressed still further the lowered oxygen uptake produced by iodoacetic acid; even in neutral solution, iodoacetate and lactate together do not give so great an uptake as that recorded in the absence of both.

(d) *Sodium azide.*

Sodium azide (NaN_3) is known to have a pronounced inhibiting action on catalase (Keilin and Hartree, 1934). In these experiments it was found to inhibit oxygen uptake to a marked degree in the standard acid phosphate solution; under these conditions the maximal inhibition was attained already at a concentration of M/3,000, and M/30,000 azide gave a considerable inhibition. In alkaline solution (M/30 K_2HPO_4), on the other hand, the effect was much reduced, M/300 azide giving 60 per cent. inhibition only instead of over 90 per cent. as in acid solution. The results are given in Table VIII; as before, some of the experiments were performed on tobacco plants.

TABLE VIII

Effect of Sodium Azide on the Oxygen Uptake of Healthy Tissue

Concentration of azide.	Solution in.	Type of plant.	Oxygen uptake (μl./gm./hr.). Control.	Oxygen uptake (μl./gm./hr.). Treated.	Inhibition. per cent.
M/300	Acid phosphate	Old tomato	70	4	94
M/3,000	„	Old tobacco	85	6	92
„	„	Young tomato	170	24	86
M/30,000	„	Old tobacco	85	49	42
M/300,000	„	„	84	81	0
M/300	Alkaline phosphate	Young tomato	144	55	61
M/30,000	„	„	125	114	(9) (not sig.)

The action of sodium azide was reversible; tobacco stem tissue treated with M/3,000 sodium azide for an hour, then washed and allowed to respire in fresh phosphate, showed a recovery of 35 per cent. When M/300 azide was used, no recovery on washing was observed; but as very dilute azide gives a considerable inhibition, M/300 is probably too strong a solution to be adequately removed by washing.

(e) *Malachite green.*

Malachite green is typical of the basic dyestuffs, which are known to be powerful inhibitors of the dehydrogenases (Quastel and Wheatley, 1931). When applied to tobacco stem tissue it was found to have a powerful inhibiting action on the oxygen uptake at extraordinarily low concentrations. It was the only substance used which gave a considerable inhibition at a concentration as low as 1/300,000 (about M/100,000).

(f) *Narcotics.*

The narcotics urethane and phenyl urethane are known to inhibit dehydrogenase systems, but only in relatively strong concentrations (Warburg, 1930; Sen, 1931). With tobacco tissue quite strong solutions gave only partial inhibition of oxygen uptake; in fact with phenyl urethane the insolubility of the substance made it difficult to get a solution strong enough to show any pronounced action, and a saturated solution with some undissolved solid still present was added to the stem slices.

TABLE IX

Effect of Washing Tissue treated with Sodium Azide (Healthy Plants)

Concentration of azide (in M/30 KH_2PO_4).	Type of plant.	Oxygen uptake (μl./gm./hr.). Control.	Treated (unwashed).	Treated (after washing).
M/300	Old tomato	33	0	4
,,	Young tobacco	130	3	4
M/3,000	Old tobacco	93	10	33

TABLE X

Effect of Malachite Green on the Oxygen Uptake of Tobacco Tissue

Concentration of malachite green.	Type of plant.	Oxygen uptake (μl./gm./hr.). Control.	Treated.	Inhibition per cent.
1/3,000	Young healthy	109	23	79
1/30,000	,,	104	39	61
1/300,000	,,	114	54	44
1/3,000,000	,,	111	105	(5) (not sig.)

TABLE XI

Effect of Urethanes on the Oxygen Uptake of Tobacco Tissue (Healthy)

Substance.	Concentration.	Oxygen uptake (μl./gm./hr.). Control.	Treated.	Inhibition per cent.
Urethane	M/3	85	30	64
,,	M/30	79	71	(10) (not sig.)
Phenyl Urethane	saturated	93	22	76
	M/300	110	75	31

(g) *Amyl alcohol.*

The vapour of amyl alcohol has been found to have a stimulating effect on the carbon-dioxide output of apples (Caldwell, 1931). When added to the solution in the Barcroft manometer vessel, a strong solution of iso-amyl alcohol (1/30) had a very strongly inhibiting effect on oxygen uptake; the control samples of tomato stem tissue had an average oxygen uptake of 126 μl./gm./hr.; in presence of 1/30 iso-amyl alcohol this was reduced to 5μl./gm./hr., an inhibition of 96 per cent. A much weaker solution (1/3,000) had no effect, neither stimulating nor inhibiting; the average oxygen uptake of both control and treated material was 97 μl./gm./hr.

DISCUSSION

The substances applied to the tomato tissue in these experiments were all known to be poisons with an inhibiting effect on one or more enzyme systems. They were all found to affect the oxygen uptake of the tissue in substantially the same way; they gave an inhibition increasing with their concentration (in some cases to a limiting value, e.g. cyanide), and if the concentration was reduced below the point where inhibition occurred, no effect was produced. Now it is well known (see e.g. Irving, 1911, Hanes and Barker, 1930) that poisons applied to green plant material as vapour in the air respired have an inhibiting effect upon respiration at high concentrations, but a stimulating effect in very small doses. With the very different technique employed in our experiments, where the poisons were applied in solution to cut material, no stimulation of oxygen uptake was observed to result from the application of smaller doses than those which produced inhibition. This point is further illustrated by the case of amyl alcohol, which stimulates the carbon-dioxide output of apples when applied as vapour (Caldwell, 1931), but in solution was found to have no effect on oxygen uptake at a low concentration and to inhibit it almost completely at a high concentration.

The effects observed on the addition of individual poisons to tomato stem slices show a very close resemblance, both in kind and in degree, to results obtained by other workers with animal tissues and bacteria.

The results which we have obtained with cyanide are very strikingly similar to those recorded by Dixon and Elliot (1929) and van Heyningen (1935) on the action of cyanide on the oxygen uptake of slices of various animal tissues. As in animal tissue slices, about 85 per cent. of the total oxygen uptake is accounted for in plant tissue slices by a system (the 'atmungsferment' of Warburg) which is reversibly poisoned by cyanide. The results obtained on the addition of cyanide in presence of added glucose agree with those of Genevois (1929), and might be regarded as confirming the conclusion he draws from them—that the oxidation of carbohydrate is effected only by that part of the respiratory system which is sensitive to cyanide. It is possible, however, that the small fraction of the respiratory enzyme system which is resistant to cyanide is also concerned with the oxidation of glucose, but is saturated by a very minute quantity which would presumably be present even in conditions of extreme starvation, and the addition of more glucose would have no effect on the oxygen uptake.

The analogy between the respiratory systems of animal and plant tissue must not be pushed too far; the results obtained on the addition of fluoride and iodoacetic acid to tomato tissue illustrate this point. Both these reagents inhibit to a marked degree the oxygen uptake of plant as well as of animal tissue. But in animal tissue they produce their effect by interrupting a process —the conversion of glucose to lactic acid—which probably does not take place in plant tissue (see Genevois, 1929). This conclusion is supported by the observation that the inhibition of oxygen uptake produced in tomato tissue by iodoacetate was not removed by the addition of lactate, which removes the inhibition observed with chicken embryos in presence of iodoacetic acid (Needham, 1932). It is of interest to note that the slowness of penetration into tissues exhibited by iodoacetic acid in Needham's experiments was observed by us at certain concentrations, see the curve for M/10,000 iodoacetic acid in Fig. 3.

The marked inhibition of oxygen uptake in tomato tissue produced by sodium azide, which has been shown by Keilin and Hartree (1934 and 1936) to act as a specific inhibitor of catalase, may indicate that catalase is an essential part of the respiratory system in plants (see Stiles and Leach, 1932). The fact that azide has a more powerful inhibiting action in acid solutions (Keilin and Hartree, 1936) has also been observed in our experiments.

The results obtained with malachite green and with urethanes are very similar to those formerly recorded (Quastel and Wheatley, 1931; Sen, 1931) with animal tissues and with bacteria.

Taken together, the effects of poisons recorded in this paper show the very great similarity that must exist between the cellular respiration of green plants and that of animals, and also of colourless plants such as yeast.

SUMMARY

Substances known to inhibit enzyme action were added to slices of tomato stem tissue, and their effect on the oxygen uptake of the tissue was measured. All the substances showed an inhibiting action which increased with their concentration. Concentrations lower than those which inhibited oxygen uptake were found to have no stimulating effect.

Cyanide (M/300) produced a reversible inhibition of about 85 per cent. of the total oxygen uptake; no greater inhibition was produced by M/30 cyanide than by M/300.

Sodium fluoride and iodoacetic acid had an irreversible inhibiting action, and sodium azide a reversible one stronger in acid than in alkaline solution.

Malachite green was effective in very small doses, but the urethanes only in high ones.

Amyl alcohol was ineffective at 1/3,000, but produced almost complete inhibition at 1/30.

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A Study of the Growth of Penicillium carmino-violaceum Biourge in Media containing Ethyl and other Alcohols, with a Note on the Production of Pigment by this Mould

BY

L. KRAUSE

AND

M. ELLIS

(*University College, Nottingham*)

With one Figure in the Text.

INTRODUCTORY

THE Penicillium used in this investigation was isolated from plant material which had originally been preserved in 10 per cent. alcohol. Evaporation had obviously occurred, leaving a lower percentage of alcohol and permitting the mould to develop. It was decided to determine the exact percentage of alcohol which could inhibit the growth of the mould, and since the question of the utilization by moulds of alcohol as a source of carbon also arose the following experimental work resulting.

A culture of the fungus was sent to Dr. Charles Thom, of the U.S. Department of Agriculture, who identified it as *Penicillium carmino-violaceum* Biourge. A detailed description of *P. carmino-violaceum* Dierckx, and of Biourge's strain of this mould, appears in Thom's monograph 'The Penicillia' (1930). Noticeable macroscopic features are the bluish-grey-green velvety appearance of the colonies, the vinaceous drops exuded by the mycelium, and the pigment which colours the reverse of the colonies, and diffuses into the medium. This pigment is at first yellow, later becoming orange, and finally dark red. In liquid media the mycelium is submerged during the earlier stages of growth, but soon forms a surface mat. When the surface of the liquid is covered, growth continues, causing the mat to become buckled and contorted. If the culture is disturbed, the first mat tends to sink into the medium, and further surface mats of mycelium may be formed. Pigment production is very conspicuous in most liquid media.

HISTORICAL SURVEY

Comparatively little detailed investigation appears to have been made into the question of alcohol toleration by moulds, though the effect of alcohol

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upon the growth of bacteria, yeasts, and torulae has been discussed in many papers. The earliest mention of such work upon moulds with which the present writers are acquainted is Raulin's account of his studies on *Aspergillus niger* in 1870. This work is fully described by Duclaux (1898) in his 'Traite de Microbiologie', and it was this description which was consulted in the present case. Raulin used a complex liquid medium containing sugar-candy as the chief source of carbon, but tartaric acid was also present to ensure acidity and to prevent the growth of bacteria. (It was pointed out that tartaric acid could also be utilized as a source of carbon, and was suggested that for experiments relating to the substitution of carbon sources the tartaric acid might be replaced by small quantities of sulphuric acid.) Raulin found that if the sugar in the medium was replaced by an equivalent weight of ethyl alcohol, germination was less active than in the medium with the sugar entirely omitted. He noted, however, that the adult mycelium was able to utilize alcohol as a source of carbon, and that if alcohol was added to an established culture higher percentages could be tolerated, and growth even appeared to be stimulated. Concentrations up to 6–8 per cent. of alcohol in the medium were tolerated. He also noted that 'as one goes up the series of alcohols, one finds that the antiseptic dose for the spores, and the nutritive dose for the mycelium, diminish as the molecular weight of the alcohol increases. With butyl alcohol, and even more with amyl alcohol, the effect is immediately toxic even to the adult plant.' In this investigation very little precaution against contamination of the cultures was taken, and no effective precaution against the evaporation of the alcohol.

Lesage (1895) found that concentrations of ethyl alcohol of 6·2 per cent. and upwards inhibited germination of spores of *Penicillium glaucum* sown upon drops of gelatin on glass slips, and suspended over water, with a small open tube containing the alcoholic solution placed at the bottom of the vessel. If the tube contained pure methyl, ethyl, butyl, amyl, or allyl alcohol, no germination took place. With 3·1 and 4·1 per cent. solutions of ethyl alcohol, germination occurred on the first and second days respectively, but although growth continued for a long time, it differed markedly from normal growth. With a series of solutions of ethyl alcohol ranging from 6·2 per cent. upwards, no germination took place. In 1897 Lesage published the results of further experiments showing the effects of alcohol vapour upon spores of *P. glaucum*. The cultures were suspended as before over alcoholic solutions, and again he found at the end of eleven days that the concentration inhibiting germination lay between 4·15 and 6·25 per cent. In other series of cultures kept for longer periods, the inhibitory concentration was found to lie between 6 and 8 per cent. When the spores were sown directly upon the alcoholic solutions, the inhibitory concentration varied about 6 per cent. Lesage suggested that the inhibition of germination of spores suspended over alcoholic solutions might be due to two factors, firstly, insufficiency of moisture, and secondly, a specific toxic action of the alcohol vapour. By placing pure water and the alcoholic solutions in separate vessels at the bottom of the flasks in which the cultures were suspended, he increased the humidity of the atmosphere in the culture flasks and succeeded in obtaining germination of spores in the presence of concentrations of up to 15 per cent. of alcohol. Higher concentrations inhibited germination and finally killed the spores. The fact that the spores were actually killed by exposure to alcoholic vapour, although they can survive long periods in the dry state, showed that there must also be a specific toxic action of the alcohol vapour. This toxic action is more marked if the temperature is raised.

Hasselbring (1908) investigated the carbon assimilation of a strain of *P. glaucum*. He used a medium containing NH_4NO_3, KH_2PO_4, and $MgSO_4$, to which various sources of carbon, including ethyl alcohol, were added. Cultures were made in 200 c.c. Erlenmeyer flasks, each with 50 c.c. of the medium, which were sterilized prior to the addition of 0·46 gm. of alcohol. These were inoculated with a spore suspension, and grown at 28° C. for ten days. The yield of dry material was used as an indication of the effect of the nutrient given. In the case of the alcohol cultures, an increase in weight took place up to the tenth day, after which there was a gradual loss in weight. Hasselbring found that alcohol was not only favourable for growth but also permitted abundant germination of spores, and considered that all concentrations which would permit growth would also permit germination. He noted that although the fungus grew vigorously in cultures containing alcohol no spores were produced.

Perry and Beale (1920) found that *Penicillium glaucum* would grow in concentrations of up to 8 per cent. of alcohol, while *Saccharomyces cereviseae* could tolerate up to 11 per cent. The medium used was dextrose broth, and the tubes were capped. No living organisms were found in concentrations above 14 per cent., while 17 per cent. alcohol in uncapped tubes completely destroyed *P. glaucum*.

Coupin (1927) investigated the nutrition of *P. glaucum*. He used an elaborate basic medium, adding in turn various organic compounds. He found that ethyl alcohol was assimilated, but not methyl, propyl, allyl, or hexyl alcohols.

METHODS

In the present investigation stock cultures were grown upon 2 per cent. malt agar or upon Czapek's agar. Both these media give abundant growth and a characteristic appearance. A number of liquid media were tried, including Czapek's solution, Richards' solution, Coons' solution, and a glucose-asparagin medium. Growth in most of these media seemed equally satisfactory, and Richards' solution was selected for use in the experimental work, being simple to prepare and easy to sterilize. The formula is as follows: Cane sugar 50 gm., KNO_3 10 gm., KH_2PO_4 5 gm., $MgSO_4 \cdot 7H_2O$ 2·5 gm., distilled water 1,000 c.c.

Flasks of 300 c.c. capacity were cleaned with potassium bichromate and concentrated sulphuric acid to remove all traces of organic matter, then washed in running water for twenty-four hours, and finally rinsed three times in freshly distilled water. The flasks were then plugged with cotton-wool and sterilized in the autoclave. The medium was made up in large flasks cleaned in the same way, and sterilized at 15 lb. pressure for fifteen minutes.

For each culture, 50 c.c. of the sterilized medium were run into a prepared 300 c.c. flask by means of a sterile pipette, care being taken to avoid accidental contamination. If alcohol was to be added to a particular culture, the necessary amount of the medium was removed with a sterile graduated pipette, and the corresponding quantity of alcohol added. Inoculations were made from suspensions of spores in sterile water, the spores being obtained from stock cultures on solid media. For each series of cultures a suspension was well shaken to ensure uniformity, and all flasks of the series were inoculated with uniform quantities of this suspension.

In the case of cultures containing definite percentages of alcohol it was obviously necessary to devise some effective means of sealing to prevent evaporation. This problem was discussed by Wirgin (1903) in a study of the inhibitory effect of ethyl alcohol upon the growth of various species of bacteria. He recommended the use of rubber caps fitting tightly over the mouths of the flasks, or of corks covered with tin foil. He admitted, however, that the rubber caps were not absolutely satisfactory even when first quality rubber was used, and stated that if corks were to be used they must be carefully selected from corks of the best quality.

Neither of these methods being entirely satisfactory, it was decided in the present work to seal the flasks with paraffin wax. After inoculation, the plugs of those cultures containing alcohol were cut off level with the neck of the flask, pushed slightly down, and melted paraffin wax poured on. A second layer of wax was added as soon as the first had set. This method proved quite satisfactory, giving an efficient seal, while the flasks could, if necessary, be opened and resealed without difficulty. The possible inhibitory effect of sealing upon growth was fully realized, but sealing is essential if the percentages of alcohol in the medium are to be kept constant for several days or longer. Cultures with as much as 10 per cent. alcohol added will develop after a few weeks if left unsealed. Control cultures without added alcohol, but sealed in the same way, were set up for comparison with each series containing alcohol, to ensure that any retardation of growth or other effects noted were due to the alcohol, and not merely to the sealing of the flasks.

All cultures were kept on the laboratory bench, at an average and fairly constant temperature of 18° C. and exposed to light though not to direct sunlight.

Experimental Results

1. *The effect of ethyl alcohol upon germination and growth.*

The first series of cultures was set up to determine the percentage of alcohol necessary to inhibit germination of spores, and contained respectively 1, 2, 3, 4, 5, and 6 per cent. by volume of absolute alcohol. A control culture without alcohol was also made, and all the cultures sealed. As in every other case, this series was set up in duplicate.

In the control culture, and in those containing 1, 2, and 3 per cent. of alcohol, germination took place and visible growth appeared in four days. A mat of mycelium soon spread over the surface of the medium, showing characteristic guttation of reddish-yellow drops, while the pigment produced by the mould coloured the medium at first yellow, turning to deep claret red in about twenty-eight days. Spores were abundantly produced. In cultures with 4 per cent. alcohol no visible growth appeared until the seventh day. The mycelium developed slowly and appeared to be stunted, but some sporulation occurred during the third week, and the pigment was produced in sufficient quantity to turn the medium a deep orange colour in twenty-eight days. In cultures with 5 per cent. alcohol no sign of growth appeared for thirty-one days, when a few very small submerged specks of sterile white mycelium were seen. These increased slightly in size and number during the next few weeks, and a few sterile surface colonies appeared, but no surface mat of mycelium was formed, no sporulation occurred, and none of the characteristic pigment was produced. With 6 per cent. alcohol, no growth appeared in two months, and on microscopic examination no sign of germination of the spores was seen. These cultures were still sterile at the end of six months.

A second series of cultures was then set up on Richards' solution with 4·5, 5, 5·5, and 6 per cent. of alcohol. The cultures containing 5 and 6 per cent. alcohol gave the same results as in the previous experiment. The cultures containing 4·5 per cent. alcohol showed macroscopic signs of germination in seven days, and presented much the same appearance as the cultures with 4 per cent. alcohol, though growth, pigment production, and sporulation were even more scanty. In those cultures with 5·5 per cent. alcohol no growth appeared, though the cultures were kept for six months.

Several repetitions of this experiment gave the same results, from which it may be concluded that the effect of alcohol in concentrations above 3 per cent. in Richards' solution is deleterious to the growth of the fungus. Concentrations of 4 and 4·5 per cent. retard germination and have an adverse effect upon growth and sporulation; a concentration of 5 per cent. retards germination very considerably and permits only scanty growth, no sporulation, and no production of pigment; while concentrations of 5·5 per cent. and over are completely inhibitory.

To determine the effect of these concentrations upon adult mycelium as distinct from the effect upon germination of spores, flasks containing 50 c.c. of Richards' solution with 5·5 and 6 per cent. alcohol were inoculated with large, healthy colonies taken from a culture on Richards' solution, and the flasks were then sealed. There were no further signs of growth, though the cultures were kept under observation for three months. Similar cultures with 4 per cent. alcohol showed some growth, comparable to the corresponding cultures of the first series but rather healthier in appearance. The toxic effect of the alcohol in concentrations below the inhibitory concentration appears to be less pronounced upon mature colonies than upon the young mycelium produced from spores, but the inhibitory concentration is the same for growth of adult mycelium as for germination.

Cultures were also made upon Czapek's solution with the addition of 3 and 5·5 per cent. ethyl alcohol, together with a sealed control culture without added alcohol. The results were similar to those obtained with Richards' solution as a medium, but the control and the cultures with 3 per cent. alcohol showed scantier growth and less pigment than the corresponding cultures on Richards' solution, though unsealed cultures on Czapek's solution gave abundant growth and pigment.

At this stage cultures were set up to test the efficiency of the paraffin-wax method of sealing, and to demonstrate the necessity for sealing. Cultures were made in flasks plugged with cotton-wool, and capped, some with grease-proof paper and some with rubber caps, but not sealed, and containing Richards' solution with the addition of 5 and 10 per cent. alcohol. These were compared with corresponding sealed cultures. In the unsealed cultures with 5 per cent. alcohol growth appeared in nine to fifteen days, while in the corresponding sealed cultures no growth was visible until the thirty-first day. (Sealed cultures without alcohol showed growth in four to five days.) In the unsealed cultures with 10 per cent. alcohol growth appeared in twenty-four days in uncapped flasks, and after two months most of the capped flasks also showed some growth, while the wax-sealed flasks remained entirely sterile.

At the same time two sets of cultures were made, one set in the 300 c.c. flasks used throughout the work, and one in 2-litre flasks. In both cases 100 c.c. of Richards' solution with 6 per cent. alcohol were placed in each flask, and inoculated from a uniform spore suspension, after which the flasks were sealed. As was to be expected, the first set of cultures showed no growth after three months, but the second set showed visible growth in seven days, indicating that considerable loss of alcohol by evaporation into the atmosphere of the flasks had occurred. Comparison of the growth in these cultures with cultures of the first experiment suggested that the effective concentration of alcohol remaining in the medium in the 2-litre flasks was about 4·5 per cent., but the matter was not pursued farther. The sole object of this experiment was to demonstrate the necessity for using uniform culture flasks throughout the work, and for precise definition of the experimental conditions.

The possibility that the concentration of available carbohydrate in the medium might influence the effect of alcohol upon germination and growth was suggested, and accordingly a basic mineral solution was made up, containing the materials of Richards' solution without the sugar. From this two media were prepared, one with 10 per cent. cane sugar and one with

2·5 per cent. Cultures were made on these two media with 4, 4·5, 5, and 5·5 per cent. of alcohol. Both with 10 per cent. and with 2·5 per cent. of sugar, the inhibitory concentration of alcohol proved to be the same as with 5 per cent. sugar (normal Richards' solution). It was noticeable, however, that the cultures with 2·5 per cent. sugar and 4 and 5 per cent. alcohol were much healthier than the corresponding cultures with 10 per cent. sugar, and even than those with 5 per cent. sugar. Those upon 2·5 per cent. sugar with 4·5 per cent. alcohol, for example, germinated in six days and showed perfectly normal growth, the mycelium forming the usual greyish-green buckled surface mat with abundant spores and the characteristic drops, while the medium became deep red in colour. Cultures on 5 per cent. sugar with 4·5 per cent. alcohol germinated in seven days, but were decidedly less healthy-looking, though spores and pigment were produced, while the cultures with 10 per cent. sugar and 4 per cent. alcohol showed abundant but unhealthy growth, the mycelium being colourless, contorted, and sterile, and the medium being coloured deep yellow instead of the usual dark red.

It appears from these results that while small variations of the sugar content of the medium do not alter the inhibitory concentration of alcohol, the toxic effects of alcohol are more pronounced in the higher concentrations of sugar. Since, however, control cultures on the two media without added alcohol showed that the medium with 10 per cent. sugar does not permit such abundant and healthy growth as that with 2·5 per cent. sugar, it seems more than probable that staling effects due to the high concentration of sugar are involved.

Using the mineral solution without sugar as a basis, the following media were made up:

R—mineral solution with 50 gm. sucrose per litre (normal Richards' solution).
R_1—mineral solution with 25 gm. sucrose per litre
R_2— ,, ,, ,, 12·5 ,, ,, ,,
R_3— ,, ,, ,, 6·25 ,, ,, ,,
R_4— ,, ,, ,, 3·12 ,, ,, ,,
R_5— ,, ,, without sucrose

Two cultures on each medium were made, and to one of each pair of cultures 4 per cent. alcohol was added. The cultures were then sealed and kept under observation for twelve weeks. At the end of this time their appearance was as follows:

R and *R+4 per cent. alcohol* as in previous experiments.

R_1. Mycelium thick, normal. Medium deep orange colour.

R_1+*4 per cent. alcohol.* Mycelium more abundant than on R_1. Medium deep red.

R_2. Mycelium less abundant than on R_1. Medium orange.

R_2+*4 per cent. alcohol.* Less growth than on R_1, but more than on R_2 without alcohol. Medium deep orange.

R_3. Less growth than on R_2. Medium orange-yellow.

R_3+4 per cent. alcohol. Less growth than on R_2+4 per cent. alcohol, but more than on R_3 without alcohol. Medium deep orange.

R_4. Very poor growth. Medium pale straw-yellow.

R_4+4 per cent. alcohol. Growth poor, but far more abundant than on R_4 without alcohol. Medium orange.

R_5. No visible growth.

R_5+4 per cent. alcohol. Slight growth, though mycelium rather stunted. Medium pale yellow.

A striking difference was thus seen between the two series of cultures. In those with very low concentrations of sugar and without added alcohol, growth was scanty, whereas in corresponding cultures containing 4 per cent. alcohol growth was much more abundant, indicating that in these cultures the alcohol was serving as an additional source of carbon. Two repetitions of this experiment produced similar results.

A further series of cultures was then made, using the basic mineral solution without sugar (medium R_5), with the addition of varying percentages of alcohol. Cultures on the mineral solution with 1, 2, and 3 per cent. alcohol showed visible growth by the twelfth day after inoculation, and after thirty days there was an abundant mat of mycelium, with sufficient pigment production to colour the medium red in the first two cases. With 3 per cent. alcohol the mycelium was rather less abundant, and the medium was coloured orange. Cultures with 4 per cent. alcohol showed no growth until the nineteenth day, and after six weeks the mycelium was still scanty, with very little pigment produced, while those with 5 per cent. alcohol showed no growth until the thirty-second day, after which a few white, submerged colonies appeared. Cultures on medium R_5 with 5·5 and 6 per cent. alcohol remained sterile.

These results show that in the absence of sugar, or in media with low sugar content, the fungus can utilize ethyl alcohol as a source of carbon, provided that the concentration of alcohol is sufficiently low. In concentrations of 3 per cent. and over, alcohol can only be used to a lesser extent, presumably owing to the formation of toxic products, while concentrations of 5·5 and 6 per cent. are inhibitory as in a normal medium.

While the cultures described above were developing, a control series was set up with distilled water in place of the mineral solution, and percentages of alcohol varying from 1 to 5 per cent. In no case was there any sign of germination.

2. *The effect of alcohols other than ethyl alcohol upon germination and growth.*

It was next decided to determine whether any other of the primary alcohols could be utilized by *P. carmino-violaceum* in the same way as ethyl alcohol, since Raulin found that other members of the series were toxic to *Aspergillus niger*, and Coupin found that methyl, propyl, allyl, and hexyl alcohols were not assimilated by *Penicillium glaucum*.

A series of cultures was set up on Richards' solution with 1–6 per cent. of methyl alcohol. In the cultures with 1, 2, and 3 per cent. methyl alcohol, growth began in three to four days, and continued for about four weeks, after which no further increase appeared to take place. Most of the growth was submerged, white, and sterile. A few sterile floating colonies were formed, but little pigment was produced, although in some of the cultures the medium was coloured a pale straw-yellow. The type of growth seen in similar cultures with ethyl alcohol, i.e. the thick surface mat of mycelium with abundant spores, yellowish-red drops, and profuse pigment production, was not seen in any of the cultures containing methyl alcohol, even after four months. The cultures with 4 per cent. methyl alcohol showed no sign of growth for twelve days, after which a few submerged specks of sterile mycelium appeared, increasing slightly during the next two months. After three months some of these stunted colonies were transferred to flasks of Richards' solution, where they immediately started to grow and soon appeared quite normal. The cultures with 5 per cent. methyl alcohol did not start to develop until the fifth week. Only a few small, stunted colonies appeared and no pigment was produced. With 6 per cent. methyl alcohol there was no sign of growth during two months, but some cultures put away and examined after three months showed three or four minute specks of submerged mycelium, which increased in number during the following month, indicating that growth was not completely inhibited.

This experiment was repeated using 5, 5·5, 6, and 6·5 per cent. methyl alcohol. The cultures with 5 per cent. methyl alcohol were as before. Those with 5·5 per cent. began to develop during the sixth week and soon resembled the cultures with 5 per cent., though rather scantier. The cultures with 6 per cent. methyl alcohol showed a few minute submerged colonies at the end of ten weeks, but there was very little increase during the next three months, though a few of the minute colonies transferred to Richards' solution at the end of this period showed themselves to be still capable of normal growth. The cultures with 6·5 per cent. methyl alcohol were kept for eight months, but showed no sign of growth.

From these results it can be concluded that, while the concentration of methyl alcohol inhibiting germination in the circumstances described is higher than the inhibitory concentration of ethyl alcohol, methyl alcohol has a far more deleterious effect upon growth after a certain stage has been reached, and even in concentrations as low as 1 per cent. it retards and eventually inhibits growth and pigment production.

A similar series of cultures was made using the basic mineral solution R_5 with methyl alcohol. These were somewhat slower in germination than those upon Richards' solution with methyl alcohol, but in ten days the cultures with 1, 2, and 3 per cent. methyl alcohol showed some growth, though less than in corresponding cultures of the previous series. In these, too, growth proceeded slowly for about four weeks, and then apparently ceased, though transference of the stunted colonies to Richards's solution at the end of four months showed them to be still capable of normal growth. The cultures with 5, 5·5, and 6 per cent. methyl alcohol were similar to corresponding cultures on Richards' solution with methyl alcohol, though scantier. In this series, as in the previous series, 6·5 per cent. methyl alcohol was found to be completely inhibitory.

The results obtained from this series indicate that while methyl alcohol can, to a slight extent, be utilized by the fungus as a source of carbon, its toxic effect upon growth makes it far less suitable for the purpose than ethyl alcohol. This toxic effect is probably due, at any rate in part, to the production of formaldehyde when the methyl alcohol is broken down by the fungus, this being far more toxic than the acetaldehyde which would be produced in cultures with ethyl alcohol.

Further series of cultures were made using Richards' solution and also R_5 with the addition of 1, 1·5, and 2 per cent. of various other simple aliphatic alcohols. One per cent. of propyl, *n*-butyl, and amyl alcohols all proved definitely inhibitory, but while spores transferred, after one month in the butyl and amyl alcohol cultures, to Richards' solution proved to be no longer viable, a few of those transferred from 1 per cent. propyl alcohol were still capable of germination and normal growth. A culture with 1 per cent. propyl alcohol which had not been re-sealed after opening showed a few specks of mycelium a few weeks later. Some cultures were accordingly made up on medium R_5 with 1, 0·5, and 0·4 per cent. propyl alcohol. These were left unsealed, and after nine days all had germinated, while in the last (0·4 per cent.) several white, stunted, floating colonies were formed. After three months all the unsealed cultures showed a thin surface film of mycelium, but this was very sparse and entirely sterile, and no pigment was produced. Propyl alcohol can therefore be utilized by the fungus, but only when present in minute traces. In sealed cultures with 1 per cent. propyl alcohol this concentration is completely inhibitory. A repetition of this experiment with *n*-butyl and amyl alcohols showed that these alcohols cannot be utilized at all, and are inhibitory to germination and growth even when present in very minute quantities. The addition of 1 per cent. of either of these alcohols to healthy cultures on Richards' solution completely inhibited further growth, and colonies transferred to Richards' solution after one month in these cultures proved to be no longer viable.

Raulin's contention that with increasing molecular weight the alcohols become more toxic is therefore borne out by the experiments described. In the case of methyl alcohol the inhibitory concentration for germination is slightly higher than that of ethyl alcohol, that of propyl alcohol is much lower, while even traces of butyl and amyl alcohols appear to be toxic. Methyl alcohol, however, while permitting germination in higher concentrations than ethyl alcohol, has a more pronounced effect in retarding germination, and is more toxic in its effect upon growth.

3. *The effect of various alcohols upon other species of Penicillium.*

Up to this point only *P. carmino-violaceum* was used as a subject of study, but it was decided to carry out a few experiments upon other species for purposes of comparison. Two strains which happened to be available were used, a strain of *P. glaucum* originally supplied by the National Collection of Type Cultures, and a strain of *P. citrinum* of unknown origin.

Cultures of these two moulds were made upon Richards' solution with the addition of 4·5, 5, 5·5, and 6 per cent. ethyl alcohol. For *P. glaucum* 5 per cent. ethyl alcohol proved to be inhibitory, while for *P. citrinum* the inhibitory concentration was 5·5 per cent., the cultures with 5 per cent. alcohol showing a very slight growth. In none of these cultures was growth normal, as compared with the control cultures without alcohol, and no spores were produced. Cultures with 1 and 2 per cent. ethyl alcohol showed normal growth and sporulation, as in the case of *P. carmino-violaceum*.

Cultures on Richards' solution with small percentages of methyl alcohol showed that both these fungi, especially the *P. citrinum* strain, were more tolerant to this alcohol in low concentration than *P. carmino-violaceum*, though the inhibitory concentration for germination was slightly lower (6 per cent. in both cases). Germination in 1 and 2 per cent. methyl alcohol took place within three days, and considerable growth occurred. With *P. glaucum* this took the form of a thick mat of sterile mycelium, but *P. citrinum* showed almost normal growth and sporulation in 1 per cent., and fairly abundant growth with scanty spore-formation in 2 per cent. methyl alcohol.

Spores of both moulds germinated within three days in Richards' solution with 1 per cent. propyl alcohol, giving at first a scanty submerged growth which continued until, in six weeks, there was a surface mat of mycelium in both cases. The growth of *P. glaucum* was decidedly abnormal, being yellowish-white in colour, with curious knotted masses of mycelium, and quite sterile, but that of *P. citrinum* was more normal, a few spores being formed. With 2 per cent. propyl alcohol, only submerged mycelium was formed, very scanty in the case of *P. glaucum*. The inhibitory concentration for *P. glaucum* was 2·5 per cent., and for *P. citrinum* 3 per cent. *n*-Butyl alcohol and amyl alcohol proved to be completely inhibitory to both species in concentrations of 0·5 per cent.

Cultures on medium R_5 with the addition of small amounts of methyl and of propyl alcohols, set up for comparison with similar cultures of *P. carmino-violaceum*, showed that the other two Penicillium species could utilize these alcohols more successfully as carbon sources. Cultures of *P. glaucum* with 1 and 2 per cent. methyl alcohol and with 0·5 per cent. propyl alcohol showed more abundant growth than the corresponding cultures of *P. carmino-violaceum*, though they resembled the latter in being completely sterile. Cultures of *P. citrinum* showed some sporulation in each case.

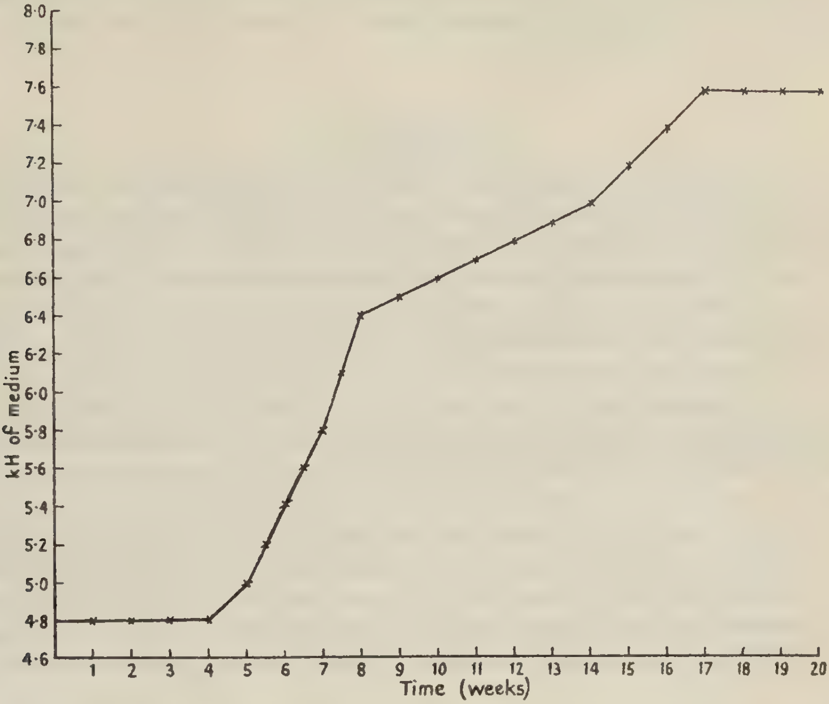
It would thus appear that as regards ethyl alcohol, the three species of Penicillium tested showed much the same reactions, the inhibitory concentrations varying only slightly. *P. glaucum* and *P. citrinum*, especially the latter, are slightly more tolerant towards propyl alcohol, and although the inhibitory concentration of methyl alcohol for germination seems to be rather lower for these species than for *P. carmino-violaceum*, the toxic effects of lower concentrations upon growth are not so pronounced.

THE PRODUCTION OF PIGMENT BY *P. CARMINO-VIOLACEUM*

It has been noted that a coloration is produced in all healthy cultures of this fungus on liquid and solid media. The colour is at first pale yellow, changing gradually to a deep claret red and diffusing through the medium. This colour-change, apart from mere intensification due to the production of more pigment, suggested to the writers that the pigment might be acting as an indicator, changing colour as the reaction of the medium altered during growth. The production of such an indicator-pigment has been described by Barber (1927) for a Penicillium of the funiculosum type.

Rough tests made with some of the medium from an old culture on Richards' solution confirmed this surmise. Cultures were then made in large flasks containing 1,000 c.c. of Richards' solution. These were left unsealed and the reaction of the medium determined at intervals by the withdrawal of 5 c.c. samples by means of a sterile pipette. In these cultures germination took place rapidly, and within a few days a thin surface mat of mycelium was formed, the mycelium being coloured a pale straw-yellow. During the next three weeks growth continued and the colour intensified to a deep golden yellow. For four weeks after inoculation, no change took place in the reaction of the medium, which remained at the initial pH 4·8. At the end of the fifth week the pH of the medium was 5·0. Samples withdrawn from the flask still appeared golden-yellow in colour. After this samples were taken twice a week, and a very slow change of reaction was observed, becoming gradually more rapid until pH 6·4 was reached eight weeks after inoculation. The change then became slower again. During this time the colour intensified considerably, but samples of the medium withdrawn showed no actual change of colour until pH 6·8 was reached, when a faint orange tint appeared. At 7·0 the samples were definitely orange in colour. After this point was reached, the change of reaction became more rapid, and pH 7·6 was reached in another three weeks (seventeen weeks after inoculation). The colour of the medium at the same time changed from orange to a deep red. No further change took place during the next two months, and growth apparently ceased. A number of old cultures were examined, some over a year old, and in all cases the reaction of the medium was found to be pH 7·6–pH 7·8. In cultures on 50 c.c. of the medium, in small flasks, the changes of reaction and colour took place much more rapidly, the medium being dark red in colour and of pH 7·6 in about three weeks.

The question of pigment production by this fungus was briefly investigated by Mr. G. E. Turfitt, B.Sc., a research student in the Department of Chemistry, University College, Nottingham. He found that the optimum pigment production occurred in a medium with the same mineral constituents as Richards' solution, but with 5 per cent. glycerol substituted for cane sugar. Two pigments were extracted from these cultures. The first is precipitated as a red-brown flocculent mass on acidification of concentrated aqueous solutions, though apparently slightly soluble in more dilute acid solutions. It is soluble in alkalis, giving a deep reddish-purple colour. Although some of this pigment diffuses into the medium in older cultures, it appears to be mainly retained in the mycelial mat. The second pigment is extractable by ether in which it is fairly soluble, and is also soluble in alcohol and acetone. It behaves as an indicator, being yellow in acid and red in alkaline solutions, the colour-change occurring at pH 7.



Graph illustrating change of reaction of medium with growth of *P. carmino-violaceum*.

The following is suggested as an explanation of the colour-changes seen in cultures of *P. carmino-violaceum*. In the initial acid condition of Richards' solution and other commonly used media, the second pigment produced by the fungus diffuses into the medium, giving a yellow colour, becoming deeper as the fungus grows and the concentration of the pigment increases. As the reaction of the medium changes, becoming neutral and finally alkaline, so the colour of the indicator-pigment changes, until the medium is bright orange-red. The first pigment is not diffused into the medium during the early stage of growth, being more or less insoluble in acid solutions, but as the medium becomes alkaline this pigment also begins to diffuse out, though to a lesser extent than the indicator-pigment. By the time the final reaction is reached (pH 7·6 in the case of Richards' solution, and pH 8·4 in the case of the glycerol medium) the medium attains an intense deep red colour due to the presence of both pigments.

SUMMARY

1. The inhibitory concentration of ethyl alcohol for *Penicillium carmino-violaceum*, under the conditions described, is 5·5 per cent., this concentration inhibiting both the germination of spores and the growth of adult mycelium. All concentrations above 3 per cent. tend to retard germination, and to have an adverse effect upon growth and sporulation.

2. The inhibitory concentration for *P. citrinum* is also 5·5 per cent., while for *P. glaucum* it is 5·0 per cent.

3. Concentrations below 3 per cent. of ethyl alcohol have no adverse effect upon growth, and allow all three species of Penicillium to sporulate freely, provided that the basic medium to which the alcohol is added is a suitable one.

4. If other more suitable carbonaceous material is scarce or absent, ethyl alcohol can be utilized as a source of carbon by all three species of Penicillium, provided that it is present only in concentrations below the inhibitory concentration for the species concerned. In low concentration it appears to be a fairly satisfactory carbon source, allowing normal growth and sporulation.

5. The inhibitory concentration of methyl alcohol for germination is slightly higher than that of ethyl alcohol for all three species, but methyl alcohol has a far more deleterious effect upon growth. It can only be utilized to a very slight extent as a source of carbon. *P. carmino-violaceum* and *P. glaucum* do not produce spores in the presence of methyl alcohol, but *P. citrinum* can sporulate in concentrations below 2 per cent.

6. Other alcohols of the aliphatic series show increasing toxicity with increasing molecular weight. Propyl alcohol can be utilized to a very slight extent as a source of carbon, but the inhibitory concentration is extremely low, especially for *P. carmino-violaceum*. The strains of *P. glaucum* and *P. citrinum* used for comparison appear to be rather more tolerant towards propyl alcohol. *n*-butyl alcohol and amyl alcohol are inhibitory even in minute quantities.

7. At least two pigments are produced during growth by *P. carmino-violaceum*, one of them acting as an indicator and changing colour as the reaction of the medium changes. The chemical nature of these pigments has not yet been investigated.

ACKNOWLEDGEMENTS

The writers wish to express their gratitude to Professor H. S. Holden, lately of University College, Nottingham, at whose suggestion this work was undertaken, and with whose advice and encouragement it was carried out.

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The Growth in Height and Weight of the Cotton Plant under Field Conditions

BY

O. V. S. HEATH

(*Empire Cotton Growing Corporation, Cotton Experiment Station, Barberton, S. Africa*)

With three Figures in the Text.

IT was pointed out by V. H. Blackman (1919) that the growth in dry weight of such annual plants as Helianthus, Cannabis, and Nicotiana conformed approximately with an exponential or 'compound interest' law up to the time of formation of the inflorescence, i.e. up to this time the relative growth rate ('efficiency index') was nearly constant. It was noted, however, that even before flowering the efficiency index fell slightly in time. Ashby (1930, 1932) found that the early growth in weight of *Zea mais* also followed the same law. Gregory (1921) found that the curve for growth in area of total leaf surface for Cucumis grown under glass in summer was approximately of the exponential form in the early stages of growth. This is the ideal form to which unconstrained growth will tend. Under constraint of an external factor at a low level, however, he found that the growth curve was approximately of parabolic form. In the former case the relative growth rate is constant, in the latter it falls with time.

It has been found (Heath, 1932) that the growth of the main axis of the cotton plant follows approximately an exponential curve from about 10 cm. height up to time of first flowering. Exponential equations of the form:

$$H = Ae^{bT}$$

were fitted, where $H =$ height in cm.; $A =$ a constant (the theoretical height at time of germination);[1] $e =$ the base of Naperian logarithms; $T =$ time in days from germination; and $b =$ the relative growth rate per day. The value found for A was in all cases too high (4 or 5 cm.), showing that the initial growth in height of the cotton seedling did not follow the same exponential course, but showed a higher growth rate due to the rapid extension growth of the hypocotyl induced by auxine and reserves stored in the cotyledons (van Overbeck, 1933). These findings have been confirmed by Afzal and Iyer (1934) in India.

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[1] Time of 'germination' was defined as the date when 50 per cent. or more of the stand had appeared above ground. Germination was generally extremely even, nearly all the plants appearing on the same day.

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Growth in total dry weight was also expected to be approximately exponential up to first flowering, and in order to compare the growth in height and weight, samples of plants were taken from the 'Normal' (control) plots of a cultivation experiment in season 1933–4 (Heath, 1937*a*) at weekly instead of the usual fortnightly intervals to give a reasonable number of observations between height 10 cm. and flowering. Figures 1 and 2 show the heights, total dry weights, and their natural logarithms plotted against time. The points represent the actual data (means of five plots, the logarithms being found from each plot mean separately), the straight lines are the mean regressions of $\log_e W$ or $\log_e H$ on time in days, and the curves are the exponential curves derived from the regressions. The exponential equations are shown in the figures. It will be seen that the points for $\log_e$ height lie approximately in a straight line from height 10 cm. up to first flowering, but that up to 10 cm. they rise more steeply. This shows the more rapid initial growth in height mentioned above, and the regression equation $\log_e H = \log_e A + bT$ has been fitted from the third to ninth occasions only (height 10·3 cm. to first flowers). For the $\log_e$ dry weights, on the other hand, the regression equation of $\log_e W$ on time has been fitted from the first to the ninth occasions, since the points all lie reasonably close to a straight line from the first sample (eight days after germination). This indicates that the relative rate of total dry weight increase (efficiency index) is approximately constant from the initial sampling up to first flowering. The value given by the equation for dry weight at time of germination[1] is 0·043 gm., and this is of the same order as the average dry weight found for the embryos of the seed used, namely 0·054 gm.[2] (cf. Ashby, 1930). Thus the growth in weight of the seedling approximates to the exponential curve from the very beginning.

By analysis of variance it is established that 99·4 per cent. of the variation in time (sum of squares between occasions) can be expressed in terms of linear regression of $\log_e$ weight on time. In spite of this, the remaining variation (0·6 per cent.) between occasions due to deviations from linear regression is also very significant ($P < 0{\cdot}01$). The time curve for $\log_e W$ is therefore very slightly but quite significantly curvilinear, and the slope (efficiency index) is not quite constant, as is indeed to be expected under fluctuating meteorological conditions. This means that in this experiment the time curve for total dry weight differs slightly but significantly from an exponential curve. Neither the first nor the ninth occasions are mainly responsible for this effect, and therefore neither should be omitted. Further analysis of the variance due to deviations from linear regression shows that the efficiency index falls slightly in time, the negative quadratic regression coefficient being found very significant. This fall is associated with a fall in the percentage of the total dry weight which is contributed by the green leaves (see Heath,

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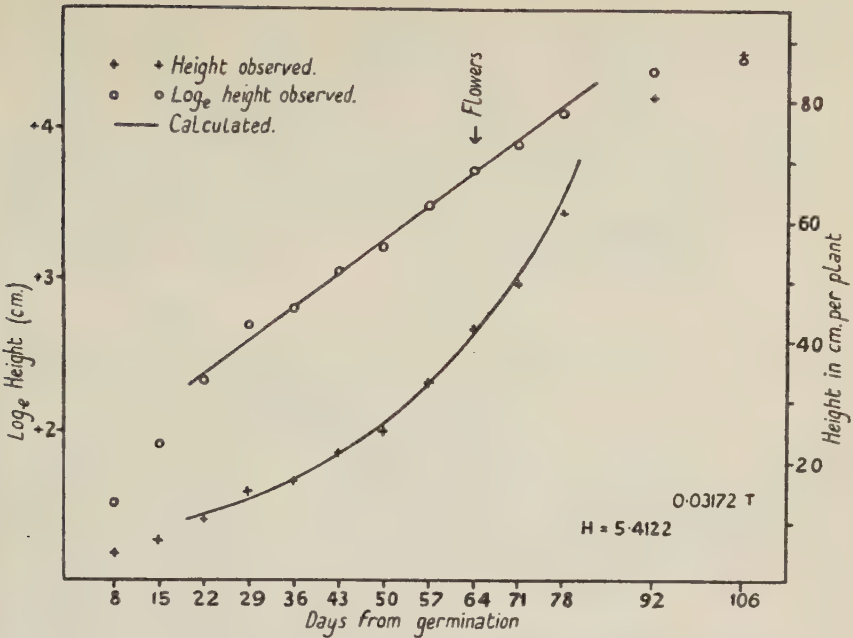
[1] See footnote, p. 515.

[2] Similar results were obtained from the fortnightly samples in 1934–5 (Heath, 1937*a*) when the equation gave a dry weight at time of germination of 0·083 gm.

FIG. 1.



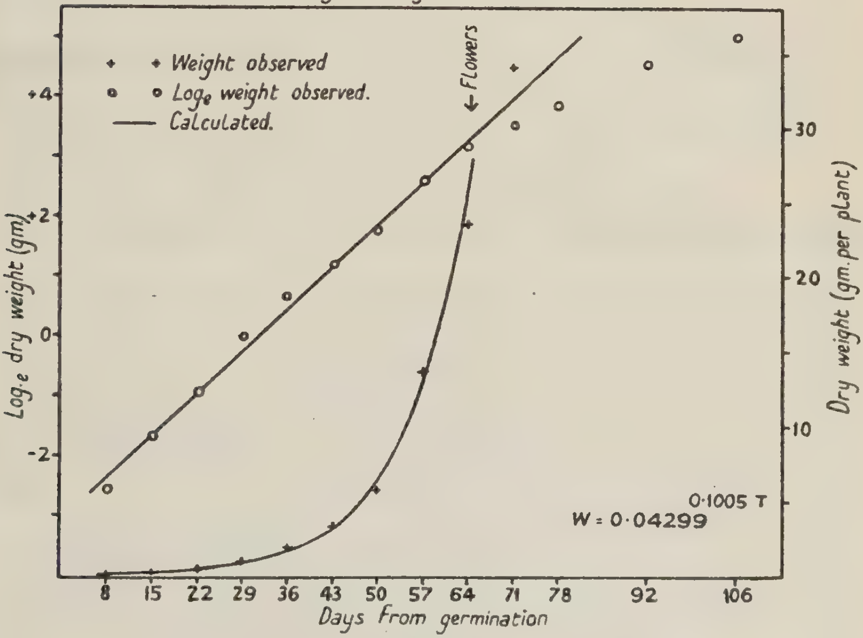


FIG. 2.

FIGS. 1 and 2. Fig. 1. Exponential growth in height of main axis, season 1933–4. Fig. 2. Exponential growth in total dry weight, season 1933–4.

1937*b*), from 77 per cent. at the initial sampling to 55 per cent. at time of flowering. The fortnightly plant sampling in 1934–5 gave similar results, the growth in weight being very nearly exponential, but the relative growth rate having a slight downward trend in time (Heath, 1937*a* and *b*) associated with a fall in leaf weight per cent. of total weight (Heath, 1937*b*).

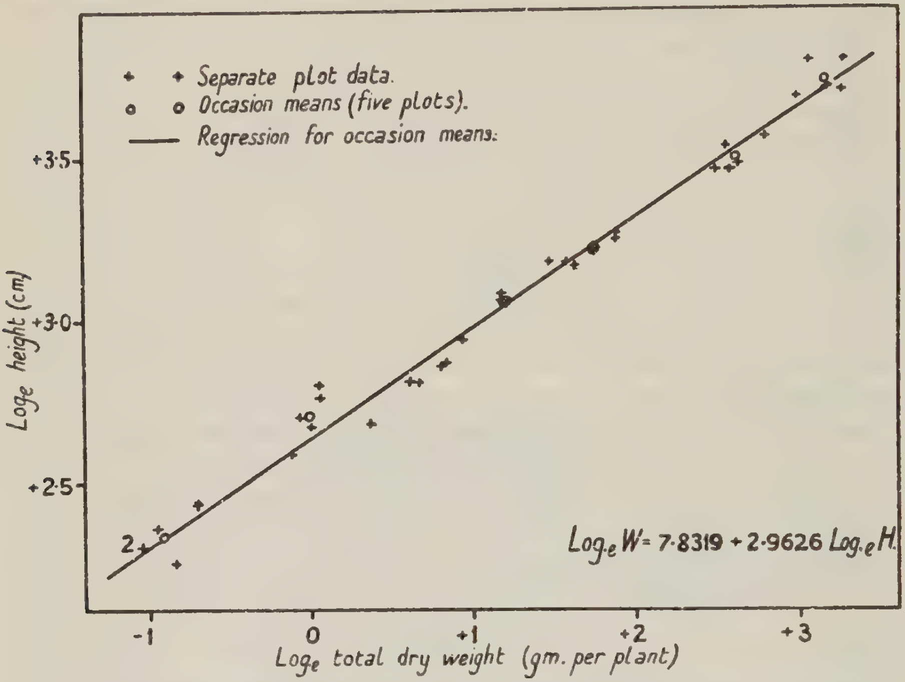


FIG. 3. Correlation of $\log_e$ total dry weight and $\log_e$ height of main axis, season 1933–4 (22 to 64 days from germination).

Similar analysis for $\log_e H$ from the third to ninth occasions shows that 98·8 per cent. of the variation in time can be expressed in terms of a linear regression. Again, the remaining variation in time (1·2 per cent.) is also highly significant ($P < 0{\cdot}01$). Therefore for height, even over the restricted range used, the time curve differs slightly but significantly from an exponential curve. The quadratic term is negligible, however, and therefore the relative growth rate has no general trend of rise or fall but merely fluctuates about a mean value.

Since growth in total dry weight and height of main stem from 10 cm. to first flowering are both approximately exponential, $\log_e H$ is bound to be highly correlated with, and give a fairly good measure of, $\log_e W$ during that period. Fig. 3, which shows $\log_e H$ plotted against $\log_e W$, indicates the close correlation between the two. The regression coefficient of $\log_e W$ on $\log_e H$ for occasion means over all plots is $+2{\cdot}9626$, i.e. $\log_e W$ increases nearly three times as rapidly as $\log_e H$, and therefore the relative growth rate in terms of weight is nearly three times as great as that in terms of height, as might be expected, since weight involves growth in three dimensions and height in one dimension only. The utility of height in the forecasting of yield has been demonstrated by Crowther (1934) for cotton and Yates (1936) for wheat.

It should be noted that as between different treatments the actual regression between height and weight may vary independently in some cases. If available, total dry weight data are to be preferred as the whole plant is included and also because the growth in total dry weight is approximately exponential from the very beginning.

SUMMARY

The growth in height and weight of the cotton plants on the control plots of a field experiment at Barberton, South Africa, in 1933–4 were estimated from random sample plants collected weekly.

The data so obtained show that from a height of 10 cm. up to time of flowering the growth of the main axis is approximately exponential. From germination up to a height of 10 cm. the growth does not follow the same exponential course but shows a higher growth rate due to rapid extension of the hypocotyl. Growth in dry weight on the other hand approximates to an exponential curve from germination until flowering.

Statistical analysis shows that in the present experiment growth both in height and weight differs slightly but significantly from the exponential course. In the former case the relative growth rate has no definite trend of rise or fall but fluctuates about a mean value; in the latter it has a slight but significant downward trend. This fall is associated with a fall in the percentage of the total weight for which the green leaves are responsible.

Values of $\log_e$ weight and $\log_e$ height are highly correlated, and the relative growth rate in terms of weight is three times as great as that in terms of height.

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Physiological Studies in Plant Nutrition

VI. The Relation of Respiration Rate to the Carbohydrate and Nitrogen Metabolism of the Barley Leaf as determined by Nitrogen and Potassium Deficiency

BY

F. G. GREGORY

AND

P. K. SEN

(From the Research Institute of Plant Physiology. Imperial College of Science and Technology, London)

With ten Figures in the Text

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INTRODUCTION

THE earlier papers in this series have dealt with the relations of certain physiological processes to deficiencies in nutrient supply. The deficiency level in these investigations was deliberately kept within moderate limits so that growth and development might continue without too great a departure from normal. In the subsequent papers a wider range of deficiency will be dealt with, and moreover the interactions of the manurial factors will

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be considered to give a wider basis for generalization. More particularly, the balance of the nutrient elements in the culture solution will be considered, and thus the specific effects of the kations as well as anions will be brought under investigation, in their relations to each other and to the major physiological processes of the plant. In the present paper the interactions of nitrogen and potassium are studied in relation to the carbohydrate and nitrogen metabolism, and the respiration rate of the leaves. As before, each leaf on the main stem is dealt with as it reaches maturity and also at later stages of senescence. In this way the changing metabolic drifts are brought into direct relation to the respiration changes throughout the life-cycle of the plant, and the data collected can be referred to precise points in the ontological cycle. The scope of the work to be covered may be gauged from a preliminary survey recently prepared by one of the authors (Gregory, 1937).

EXPERIMENTAL PROCEDURE

Cultural methods.

As in previous investigations the plant used was a pure line Barley var. 'Plumage Archer'. The plants were grown in the summer of 1932 in the open at Rothamsted Experimental Station in sand culture, the usual method being employed. To obtain the necessary material for experimental purposes 600 pot cultures were required. Sowing took place on May 6; germination began on May 16 and was practically complete in three days.

The manurial types. The manurial scheme was devised to give two series: (1) with maximal level of nitrogen and decreasing potassium, (2) with maximal level of potassium and decreasing nitrogen, and in addition one set with both minimal nitrogen and minimal potassium. In all cases the level of phosphorus and the other manurial constituents was maintained at a uniformly high level; thus, in all, six manurial combinations were used. The fully manured series N_1K_1 served as control and received nitrogen (N), potassium (K_2O), and phosphorus (P_2O_5) in the ratio of 3:2:1. The two series deficient in potassium, N_1K_3 and N_1K_5, received respectively one-ninth ($\frac{1}{9}$) and one-eighty-first ($\frac{1}{81}$) part of the potassium added to the control series. In the two nitrogen-deficient series N_3K_1 and N_5K_1 the nitrogen added was reduced in the same way to $\frac{1}{9}$ and $\frac{1}{81}$ of the maximum, while in the series N_5K_5 the level of both nitrogen and potassium was reduced to the minimum $\frac{1}{81}$ of the level in the control series. It will be noted that in the symbols used to designate the various manurial series the higher suffix numbers represent *increasing* grades of *deficiency*.

The following numbers of cultures in each manurial series were used: N_1K_1, 80; N_1K_3, 80; N_1K_5, 90; N_3K_1, 110; N_5K_1, 120; N_5K_5, 120. The actual amounts of nutrients received per pot are given below (Table I). In addition traces of iron and manganese were given. The nitrogen was added as sodium nitrate, and in the nitrogen-deficient series the level of sodium was adjusted to that of the control series by addition of sodium sulphate in requisite amount. The manures were added in five weekly doses, the first on May 17, after the seeds had germinated. The second dose was added on May 24, the third on May 30, at which time all the remaining phosphorus, magnesium, and calcium were applied. The last doses were given on June 7 and June 14 respectively, and consisted in potassium sulphate and sodium nitrate only.

The manurial treatments used resulted in large differences in development and final yield. The manurial deficiency symptoms encountered have been sufficiently described by Richards and Templeman (1936). In the following Table II data of tiller number and dry weight production for each series are given which show very clearly the large differences obtained.

TABLE I

Manuring Scheme (gm. per pot.)

Series	$NaNO_3$.	K_2SO_4.	Na_2HPO_4 12 H_2O.	$MgSO_4$ 7 H_2O.	$CaCl_2$.	Na_2SO_4 10 H_2O.
N_1K_1	7·37	1·50	2·04	1·01	0·30	—
N_1K_3	7·37	0·167	2·04	1·01	0·30	—
N_1K_5	7·37	0·019	2·04	1·01	0·30	—
N_3K_1	0·818	1·50	2·04	1·01	0·30	12·42
N_5K_1	0·091	1·50	2·04	1·01	0·30	13·80
N_5K_5	0·091	0·019	2·04	1·01	0·30	13·80

TABLE II

	Tillers per pot.	Dry wt. at harvest (gm. per pot.).			
Series	July 4.	Ears.	Stem.	Root.	Total.
N_1K_1	55·6	29·9 ±0·84	47·4 ±0·44	16·7 ±0·70	90·0 ±1·31
N_1K_3	53·1	6·63±0·27	22·0 ±0·43	5·51±0·18	34·2 ±0·67
N_1K_5	30·4	0·00	7·23±0·47	2·14±0·18	9·37±0·60
N_3K_1	21·4	4·77±0·35	8·74±0·29	4·04±0·18	17·6 ±0·64
N_5K_1	5·8	1·05±0·06	2·26±0·07	1·60±0·07	4·92±0·18
N_5K_5	5·4	0·97±0·06	2·04±0·05	1·23±0·06	4·23±0·12

Respiration apparatus.

Respiration rate in this investigation was determined by the gasometric method. The apparatus used is shown diagrammatically in Figs. 1 and 2. It was first devised, in collaboration with Dr. J. I. Armstrong, in 1931, and has since been in continuous use and has proved easy to handle and to be reliable. By means of a 'push and pull' system the pressure is maintained at atmospheric within the respiration chamber, thus minimizing risk of leak and greatly facilitating the change of the absorbers; indeed, these can be changed singly while the apparatus is running without fear of contamination of CO_2 from the air, or interfering with the remaining absorbers. A brief description of the apparatus may be given. The respiration chambers, of which there are seven arranged in parallel, consist of specimen jars (12 cm. × 12 cm. diameter, vol. 1·3 litres), with ground-in closely fitting lids and provided, as shown, with outlets above and below. The chambers are housed within a thermostat

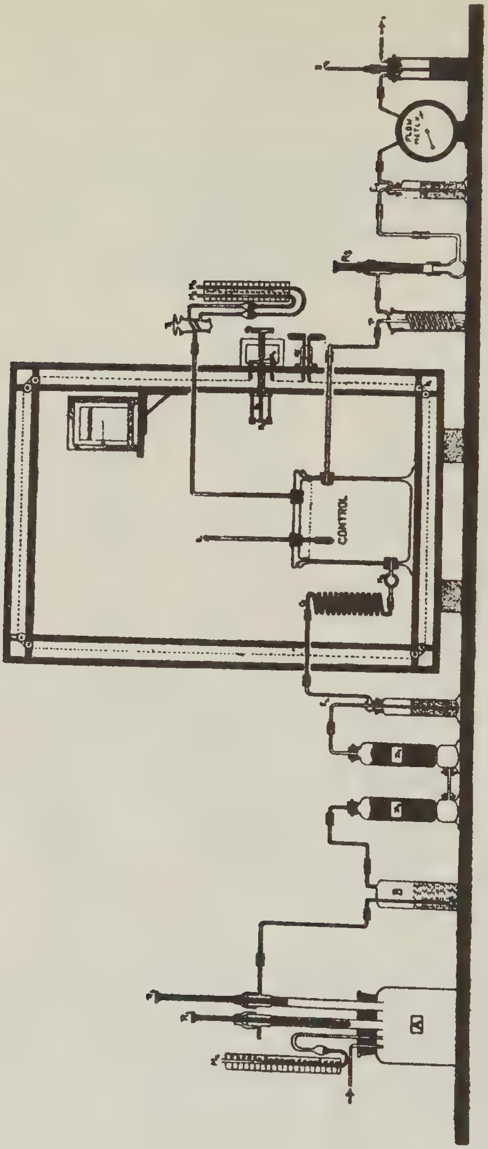

Fig. 1. Respiration apparatus in elevation: for details see text.

consisting of a double-walled wooden box, having the space between the double walls strung with resistance wire to serve for heating. By means of a Hearson capsule the temperature is controlled within 0·25° C. The air-stream from a waterblower is forced into the system through the jar *A* and

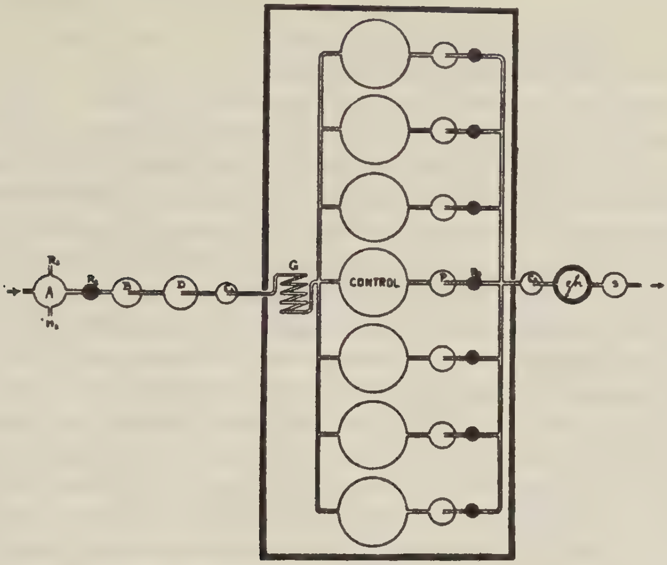

FIG. 2. Respiration apparatus in plan: for details see text.

is removed at the other end by means of a filter-pump through the Mariotte bottle *S*. The pressure in the jar *A* is indicated by a mercury manometer M_1. R_1 and R_2 are two variable capillary flow-regulators already described elsewhere (Gregory, 1933). R_1 acts as a capillary leak so that the air may be delivered into the jar at a higher rate than is required to flow through the respiration chambers, thereby making regulation by the filter-pump considerably easier. The air-stream leaves the jar *A* by the second regulator R_2 and passes thence through a scrubber *B* containing saturated sodium hydroxide solution to remove CO_2, and the towers D_1, D_2, containing calcium chloride and soda-lime respectively. The bubbler E_1 contains saturated baryta solution to act as a test for the complete absorption of CO_2. In passage through E_1 the air is resaturated with water. The air-stream now enters the thermostat, first flowing through a coil of compo tubing, *G*, to attain the requisite temperature, and then into a brass tube connected with the seven respiration chambers (see Fig. 2) arranged in parallel. From the other outlet of the chambers the air-streams pass directly out of the thermostat to a series of bubblers *P* containing soda for the absorption of CO_2. The absorbers consist of a glass spiral resting loosely on the upturned end of the tube through which the gas-stream enters. This tube is drawn on into a fairly fine jet from which the gas escapes in bubbles which pass up the spiral carrying with them the solution. Good contact between the gas and absorbing solution is thus ensured as well as adequate mixing of the solution. For gas-streams up to 5 litres per hour such absorbers are efficient. After passing through a series of flow regulators R_3, the gas-streams are collected in a second brass tube connecting together the parallel circuits. Finally, after passage through a test bubbler E_2 containing baryta, the gas flows through a gas-meter to the Mariotte bottle and thence to the filter-pump.

The central jar of the series is used as a control chamber. The lid of this chamber is provided with two holes, one carrying a thermometer and the other connected with two manometers M_1, M_2 through a two-way tap as shown. One of these manometers is filled with mercury, the other with water, and serve to measure the pressure within the system of respiration chambers.

To use the apparatus the filter-pump is first started and then the blower; meanwhile the mercury manometer is watched and the various flow resistances R_1 and R_2 are so adjusted that the pressure in the system is atmospheric. By suitably adjusting the flow resistances and the water-pumps the total flow of gas as measured by the flow-meter can be brought to any desired level, the pressure meanwhile remaining at atmospheric level within the respiration chambers. The actual rate of flow used was approximately 5 litres per hour through each chamber, in total, therefore, 35–40 litres. When adjustment is complete the two-way tap T_2 is turned to bring the water manometer into action, and the final fine adjustment is made with the flow regulator R_2 to bring the pressure in the chambers accurately to atmospheric level. The whole apparatus has been left running for several days without more attention than a slight adjustment of the flow regulators. The pressure rarely varies by more than a few millimetres of water. By means of the variable capillary resistances R_3 the rate of flow through the separate chambers can be readily equalized.

Leaf material used.

The seven chambers of the respiration apparatus allowed the leaves of the six manurial series to be investigated simultaneously, the central chamber being used as control. Each leaf in turn on the main axis was sampled as it reached maturity, and later stages were also investigated. Plants were selected at random and the leaves removed between 10 a.m. and noon, placed in stoppered glass tubes lined with moist filter-paper, and taken to the laboratory. A sufficient number of leaves of each kind were collected to give approximately 3 gm. of fresh material. As the nitrogen-starved leaves were small, while the potassium-starved leaves died so rapidly that the tip was in some cases dry before complete expansion, there was not sufficient material in the later samples to collect 3 gm. and a smaller weight had to be employed. After weighing, the leaves were mounted with their cut ends in water in glass tubes held in a carrier of paraffin wax which was placed in the respiration chamber. The air-stream was allowed to flow through the chambers at the required rate for an hour and a half to allow the leaves to reach a steady rate of respiration and to remove the air originally in the chambers.

The dates of sampling of the various leaves on their several occasions are entered in Table III.

TABLE III

Dates and Occasions of Sampling of Successive Leaves

Leaf No.	N_1K_1.	N_1K_3.	N_1K_5.	N_3K_1.	N_5K_1.	N_5K_5.
1	May 29, 31 June 2	May 28, 31 June 2	May 29, 31 June 2	May 28, 31 June 2	May 29, 31	May 28, 31
2	June 3, 5, 11	June 3, 5, 11	June 3, 5, 11	June 3, 5, 11	June 3, 5, 13	June 3, 5, 13
3	June 10, 14, 15	June 10, 14, 15	June 10, 14, 15	June 11, 14	June 13, 20	June 13, 20
4	June 14, 18	June 14, 18	June 14, 18	June 15, 20	June 20, 28	June 20, 28
5	June 18, 25	June 18, 25	June 18, 25	June 20, 28	June 28, July 9	June 28, July 9
6	June 25, 29	June 25, 29	June 25, 29	June 28, July 4	July 9, 15	July 9, 15
7	June 29, July 4	June 29, July 4	June 29, July 4	July 4, 8	July 15, 18	July 15, 18
8	July 4, 9	July 5, 11	July 8, 11	July 8, 11	July 18, 23	July 18, 23
9	July 9, 15	July 11, 17	July 17, 19	July 11, 17	July 23, Aug. 2	July 23, Aug. 2
10	July 15, 18, 20	July 17, 19	July 20, 23	July 17, 23		
11	July 18, 20					

The retardation of leaf expansion due to nitrogen deficiency necessitates later sampling in the series N_3 and N_5. Low potassium supply scarcely affects rate of leaf production.

Respiration determination.

The experiments were performed at 25° C.±0·5 and continued for six hours. In a few earlier experiments a single period of six hours was studied, but later the whole duration was divided into two periods of three hours, so that the course of respiration in the dark could be followed. At the close of the experiment the leaves were dried in a rapid current of air at 60° C.

The absorbers were filled with 50 c.c. of 4N sodium hydroxide from an automatic pipette, were inserted in place after the preliminary clearing of the apparatus of CO_2, the time noted and the experiment then started.

The absorbers, after removal from the apparatus, were washed with CO_2 free water into 250 c.c. graduated flasks. Ten cubic centimetres of 10 per cent. $BaCl_2$ solution were added to precipitate carbonate, the volume made up to the mark and the flask stoppered. After shaking to mix the contents the flask was left to stand overnight, and the precipitate allowed to settle. An aliquot part of the supernatant solution was then removed by means of an automatic pipette so that the precipitate was not disturbed nor the solution again brought into contact with air. Titration was begun with N/20 HCl and completed with N/40 HCl, using phenolphthalein as indicator. For each series of experiments a control absorber was used attached to the central chamber, which remained empty. In this way a correction was made for the original carbonate content of the soda solution. The CO_2 evolved was estimated as mg. per hour per gm. dry weight of leaf. Preliminary experiments showed an error of $\pm$0·5 per cent. in the determination of CO_2.

Sugar analyses.

Separate samples of leaves comparable with those used in the respiration determinations were collected at the same time and used for sugar analyses. Leaf material weighing 0·5–1·0 gm. was taken, accurately weighed while fresh, cut into pieces, and killed in absolute alcohol. The material was boiled in alcohol for fifteen minutes to deactivate enzymes, and then preserved in stoppered tubes under alcohol till analysed. Extraction was completed before analysis by again boiling with 95 per cent. alcohol for six hours under a reflux condenser, and later the alcohol was removed under reduced pressure at 30° C.; further treatment was similar to that described by Gregory and Baptiste (1936). The sugar estimation was carried out by the Hagedorn and Jensen (1923) method. Free reducing sugar was determined after clearing with basic lead acetate (specific gravity 1·25, 0·1 c.c. per 0·2 gm. fresh weight of leaf), and the total sugar after acid hydrolysis (1 per cent. HCl at 70° C. for 15 minutes). Sugar was estimated as mgm. hexose per gm. dry weight of leaf, the dry weight being determined from the ratio of fresh weight to dry weight in the comparable sample used for respiration.

Nitrogen analyses.

Separate samples of leaves were collected at the same time as those used for respiration, for the determination of nitrogen content. Total nitrogen, protein N, total crystalloid N, and total amino N were separately estimated by the methods described by Richards and Templeman (1936), to whose paper reference may be made for details of procedure.

The leaves, after removal from the plant, were weighed and dried in an electric oven at 50–60° C. for seven hours, while a rapid stream of dry air was passed over them. The dried samples were ground immediately before analysis in a Wiley micro-mill. Sufficient leaves were collected to give 1·5–2·0 gm. dry matter.

Total nitrogen was estimated by the micro-Kjeldahl method after previous reduction of nitrate with reduced iron. Crystalloid N was estimated by extraction of the material, after previous thorough grinding in a mortar with phenol-water, and filtering through paper pulp. Protein was removed from the extract with 50 per cent. trichloracetic acid and subsequent filtering. In the filtrate total crystalloid N was determined by micro-Kjeldahl method.

Amino N was determined on an aliquot of the cleared extract by a modifica-

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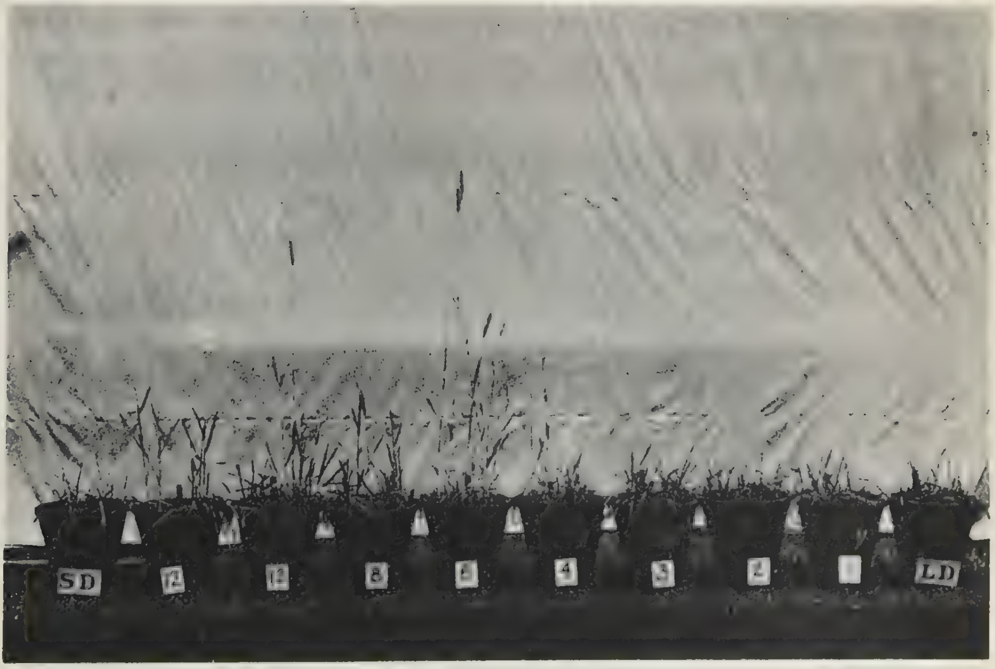

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tion of the Sörensen formol titration method. Protein N was estimated by the difference between total N and total crystalloid N.

As previous experience had shown that treatment had little effect on the sugar and nitrogen content of the first leaves all analyses began with the third leaf.

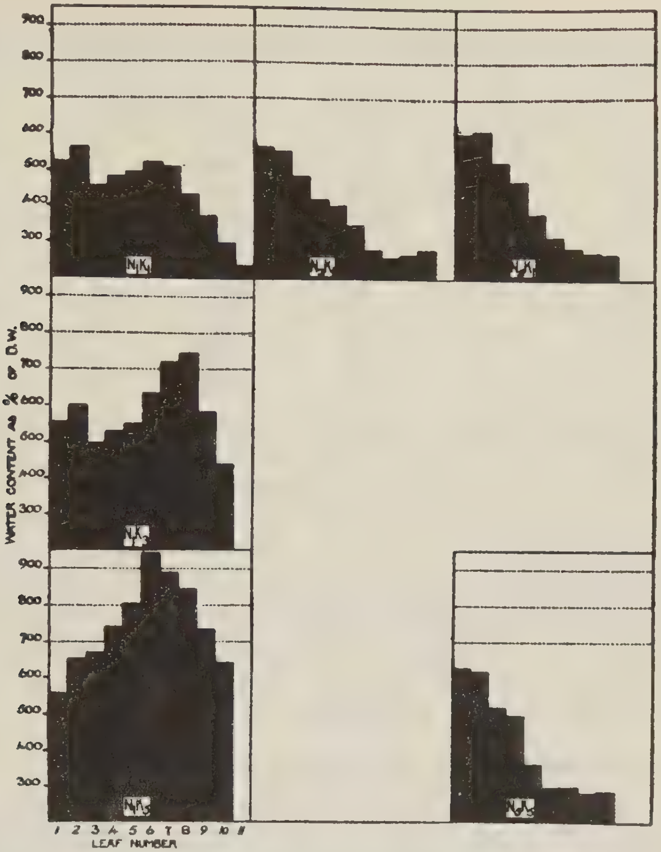

Fig. 3. Water content of successive leaves in the various manurial series, as percentage of dry weight. N_1 full nitrogen, N_3 $\frac{1}{9}$ nitrogen, N_5 $\frac{1}{81}$ nitrogen, K_1 full potassium, K_3 $\frac{1}{9}$ potassium, K_5 $\frac{1}{81}$ potassium.

EXPERIMENTAL DATA

Water content of the leaves.

Routine determinations of water content of the leaves sampled were made, and the data afforded opportunity of verifying and extending previous observations on the effect of nitrogen and potassium deficiencies on water content of leaves. The mean values of all determinations of successive leaves in the various treatments are given in Table IV, and are shown graphically in Fig. 3. The data entered are the water contents as percentage of the dry weight.

The water content varies very considerably with leaf number, the fully manured series and those with potash deficiency (N_1K_3, N_1K_5) showing a maximum water content at the sixth to eighth leaf. In the series with nitrogen deficiency (N_3K_1, N_5K_1) the water content falls progressively with each member of the leaf series. Progressive potassium starvation leads to continuous *increase* in water content, while nitrogen deficiency has the reverse effect. Simultaneous reduction in both nitrogen and potassium (N_5K_5) leads to the same water content as that due to nitrogen deficiency of the same severity in the presence of excess of potassium: nitrogen starvation thus completely masks the effects of potassium shortage. The former results of Gregory and Richards (1929) and Richards (1932) have thus been confirmed. These results are not in agreement with those recently published by Warne (1936) and as this issue has been dealt with elsewhere (Gregory, 1937) it will not be further discussed in this paper.

TABLE IV

Water Content of Successive Leaves (Water as Percentage Dry Weight)

Leaf No.	N-deficient		Control	K-deficient		N- and K-deficient
	N_5K_1.	N_3K_1.	N_1K_1.	N_1K_3.	N_1K_5.	N_5K_5.
1	602	566	523	553	559	628
2	609	554	560	599	653	618
3	527	489	458	497	669	520
4	474	424	483	532	743	496
5	383	404	494	551	805	358
6	318	349	520	636	945	298
7	288	283	500	719	891	299
8	275	260	434	745	845	274
9	271	267	376	584	735	277
10	—	270	299	439	642	—

Relation of respiration rate to manuring and leaf succession.

The rates of respiration in the potassium-deficient series are given in Table V, while the data for the nitrogen-deficient series are entered in Table VI. The results are shown graphically in Figs. 4 and 5. All values are stated as mg. CO_2 per hour per gm. dry weight. Under each treatment are entered all the values obtained, those in the first column being the respiration rates obtained on the first occasion of sampling, approximately at the time of full emergence of the respective leaves. The other columns give the values for the leaves at later stages. The second sampling of a particular leaf in general coincided with the time at which the next leaf in succession had just emerged. Details as to time of sampling have already been presented in Table III.

The following effects may be noted: (1) with regard to the respiration of successive leaves, in the fully manured (N_1K_1) and medium K-starved series (N_1K_3) respiration rises with leaf succession to reach a maximum at

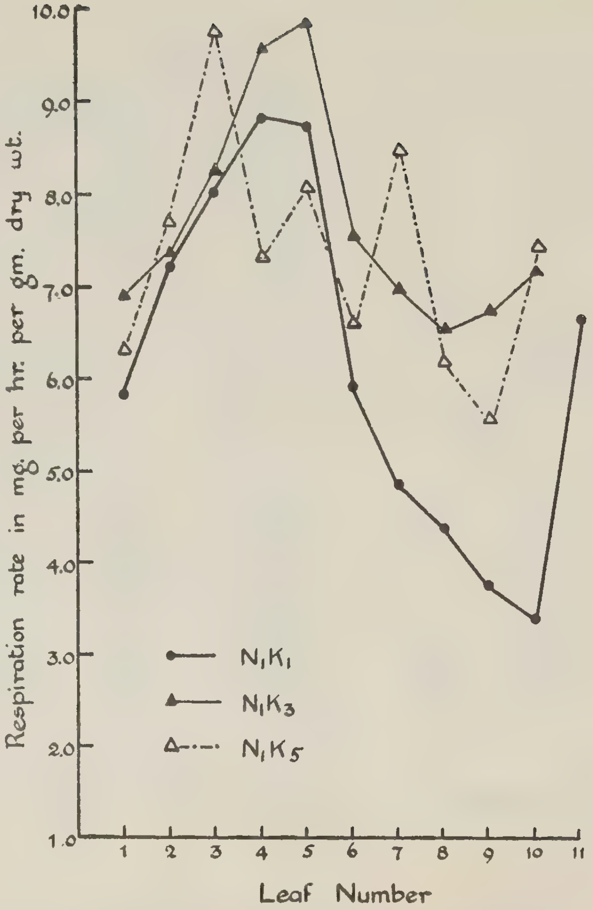

FIG. 4. Respiration rate of the control and potassium-deficient series for successive leaves at emergence. K_1 full potassium, K_3 $\frac{1}{9}$ potassium, K_5 $\frac{1}{81}$ potassium.

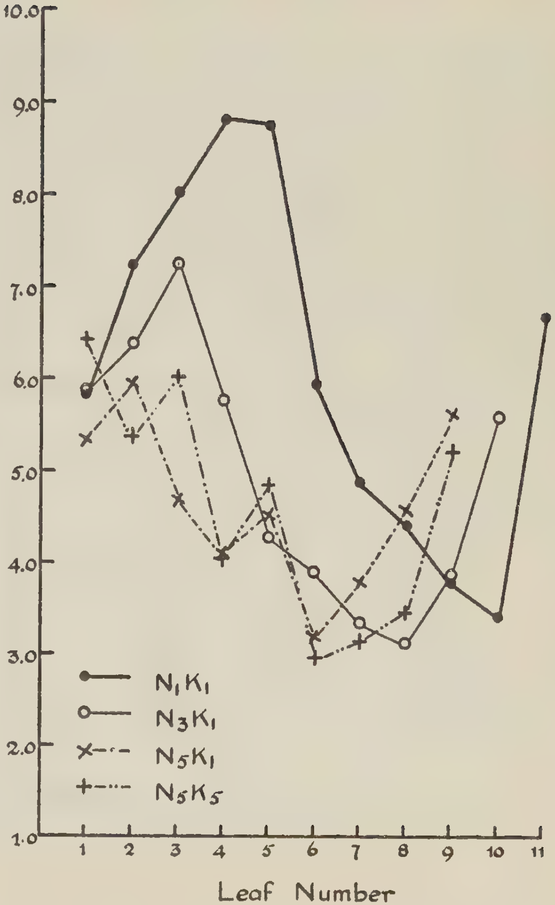

FIG. 5. Respiration rate of control and nitrogen-deficient series for successive leaves at emergence. N_1 full nitrogen, N_3 $\frac{1}{9}$ nitrogen, N_5 $\frac{1}{81}$ nitrogen, K_5 $\frac{1}{81}$ potassium.

TABLE V

Rate of Respiration of Successive Leaves at Different Times from Emergence (mg. CO_2 per hour per gm. dry weight). Temp. 25° C.

N_1K_1, N_1K_3, N_1K_5 are progressive stages of K deficiency (1:1/9:1/81).

		N_1K_1.			N_1K_3.			N_1K_5.	
Leaf No.	1st sample.	2nd sample.	3rd sample.	1st sample.	2nd sample.	3rd sample.	1st sample.	2nd sample.	3rd sample.
1	5·82	8·02	7·58	6·91	10·37	8·40	6·32	7·53	8·59
2	7·21	9·00	6·97	7·36	10·50	7·66	7·73	9·97	6·65
3	8·01	7·84	7·22	8·27	8·12	8·14	9·79	8·81	6·39
4	8·82	6·63	—	9·58	8·13	—	7·34	7·55	—
5	8·74	6·09	—	9·89	8·87	—	8·11	5·50	—
6	5·94	4·17	—	7·57	6·10	—	6·63	6·59	—
7	4·88	4·56	—	7·00	5·71	—	8·51	6·35	—
8	4·40	4·49	—	6·58	6·87	—	6·22	6·71	—
9	3·78	3·64	—	6·77	6·11	—	5·60	5·72	—
10	3·40	3·60	4·54	7·20	6·40	—	7·47	8·68	—
11	6·67	6·45	—	—	—	—	—	—	—
Mean	6·61	5·86	—	7·71	7·72	—	7·37	7·34	—

TABLE VI

Rate of Respiration of Successive Leaves at Different Times from Emergence (mg. CO_2 per hour per gm. dry weight). Temp. 25° C.

N_3K_1, N_5K_1, two stages of N deficiency (1/9:1/81).

		N_3K_1.			N_5K_1.			N_5K_5.	
Leaf No.	1st sample.	2nd sample.	3rd sample.	1st sample.	2nd sample.	3rd sample.	1st sample.	2nd sample.	3rd sample.
1	5·84	6·59	6·52	5·33	6·05	—	6·41	7·64	—
2	6·38	7·89	6·02	5·98	5·93	6·28	5·37	6·01	7·32
3	7·23	5·96	—	4·69	4·19	—	6·01	4·59	—
4	5·76	4·04	—	4·10	3·80	—	4·03	3·69	—
5	4·26	3·11	—	4·54	2·59	—	4·84	2·86	—
6	3·90	3·33	—	3·19	2·51	—	2·97	2·60	—
7	3·32	3·64	—	3·79	2·96	—	3·13	3·44	—
8	3·11	2·62	—	4·58	3·99	—	3·47	4·50	—
9	3·86	3·68	—	5·62	4·81	—	5·20	3·71	—
10	5·58	5·58	—	—	—	—	—	—	—
Mean	4·92	4·64	—	4·65	4·09	—	4·60	4·34	—

the fourth or fifth leaf. More extreme K deficiency (N_1K_5) leads to an earlier maximum at the third leaf. In the series with increasing nitrogen starvation the maximum occurs at progressively early stages: N_3K_1, third leaf; N_5K_1, second leaf; N_5K_5, first leaf, respectively. After the maximum value the respiration of later leaves declines and a minimum appears somewhere between the seventh (N_5K_1) and tenth (N_1K_1) leaf, while in the last-formed leaves respiration is again higher. (2) The effect of leaf age may be assessed from the respiration values obtained on the various sampling occasions for the same leaf. Except for the first and second leaves, which in all manurings give a higher respiration on the second occasion, in general the respiration rate declines with advancing age of the leaf, the rate of senescence being greatest in the fifth leaf in all cases (except N_3K_1 at fourth). (3) The effects of manuring are very marked. With increasing deficiency of potassium the respiration rate for comparable leaves passes through an optimum value at the level $\frac{1}{9}$ (K_3) and again falls with more acute starvation. This optimum relation is equally marked at the two occasions of sampling. In the second and third leaves on the first occasion of sampling, however, the respiration reaches the highest level in the N_1K_5 series. The potassium deficiency effect becomes more marked in the later leaves of the succession. In every respect the former findings of Richards (1932) on the effect of potassium deficiency have been confirmed.

The effect of nitrogen deficiency is to reduce respiration rate, the effect relative to the control series reaching a maximum in the fifth leaf (N_3) or the fourth leaf (N_5) and tending to disappear later. The effect of potassium deficiency at the lowest nitrogen level (N_5K_5) is apparent in the first three leaves, which give higher values than the corresponding leaves of the N_5K_1 series. In the later leaves, however, agreement between these two leaves is close, and nitrogen deficiency completely masks the effect of deficient potassium.

Change in respiration rate of leaves kept in the dark.

The drift of respiration of the leaves kept in the dark may be followed from the two consecutive three-hourly determinations of respiration rate performed on all leaves in all manurial series after the third leaf. The average values over the whole six-hour period have already been given in Tables V and VI. In Table VII the percentage decrease in respiration rate between the two three-hour periods is shown, in each case the first period being taken as the standard.

From the values in this table the following conclusions appear: (1) In the control series respiration rate falls with time to varying extents in the series of successive leaves. This effect increases in the earlier leaves, reaches a maximum at the fifth leaf, and then rapidly decreases. The later leaves show an almost constant respiration over a period of six hours. (2) In the potassium-deficient series the effect again increases with successive leaves to reach a maximum at the fifth (N_1K_3) or sixth (N_1K_5) leaf. In later leaves the effect becomes less evident. The decline in respiration is more evident in the more deficient series N_1K_5. (3) With leaves of more advanced age (second sample) the fall in respiration rate with time is less apparent than at the earliest stages of the same leaf; again the K_5 series show the greatest effect.

TABLE VII

Percentage Decrease in Respiration Rate in Two Consecutive Three-hour Periods

	Control	K-deficient		N-deficient		N- and K-deficient
Leaf No.	N_1K_1.	N_1K_3.	N_1K_5.	N_3K_1.	N_5K_1.	N_5K_5.
3. 1st sample	12·1	14·5	31·0	13·5	−2·9	11·1
2nd sample	15·6	13·1	12·2	—	—	—
4. 1st sample	—	—	—	6·0	0·5	4·3
5. 1st sample	24·2	22·9	25·8	3·9	−0·4	3·3
2nd sample	9·4	14·9	20·6	—	—	—
6. 1st sample	21·0	17·0	34·8	0·5	−4·5	1·7
2nd sample	12·6	8·2	16·4	5·9	—	—
7. 1st sample	10·3	17·7	16·7	1·8	1·6	8·0
2nd sample	9·4	5·6	5·8	−5·1	—	—
8. 1st sample	7·4	3·9	14·5	−1·9	−3·3	6·4
2nd sample	2·4	6·3	7·3	−3·9	3·9	0·9
9. 1st sample	−0·8	2·5	5·0	−0·5	−6·8	0·0
2nd sample	−1·6	3·2	—	1·6	4·3	4·0
10. 1st sample	6·6	−1·7	2·3	4·0	—	—
2nd sample	2·7	−2·0	10·0	5·9	—	—
3rd sample	1·3	—	—	—	—	—
11. 1st sample	2·2	—	—	—	—	—
2nd sample	−4·1	—	—	—	—	—

(4) In the nitrogen-deficient series only the first leaf of the series N_3K_1 shows any considerable fall in respiration; all the remaining leaves show very little fall or in many cases a slight rise in respiration during the second three-hour period. Under conditions of N starvation, therefore, respiration rate is maintained at a constant level in the dark for a period of six hours, and the same is true of the later leaves of plants receiving high nitrogen doses. Since, however, the nitrogen content of the later leaves for all manuring decreases considerably, as Richards and Templeman (1936) have shown, and as will be shown also for this experiment in a later section, the maintenance of a steady respiration rate in the dark is characteristic of nitrogen-deficient leaves. Further, the maximum fall in respiration occurs in the series N_1K_1, N_1K_3, and N_1K_5 in the early leaves, which shows the maximum respiration rate; the higher the respiration rate, therefore, the more rapid is the decline with time. This result was sufficiently interesting to warrant further investigation, and to this end two long-period experiments were carried out.

Effect of nitrogen deficiency on the drift of respiration.

The first experiment was performed with the fifth leaves of series N_1K_1, N_1K_3, and N_1K_5, which as already shown in the control series gave the maximum respiration rate, and the most marked decline with time. The duration of this experiment was fifty-four hours. The second experiment was continued for sixty-eight hours, and for this the ninth leaves of series N_1K_1, N_3K_1, and the sixth leaves of series N_5K_1, N_5K_5, were used. Hourly determinations of CO_2 were made for the first six hours and later determination for six-hour periods were made in the first experiment, and for four to eight hours in the second. The results of these experiments are given in Tables VIII and IX.

TABLE VIII

Rate of Respiration of Fifth Leaves (mg. CO_2 per hour per gm. dry weight)

Hours in darkness.	Control	Increasing K deficiency	
	N_1K_1.	N_1K_3.	N_1K_5.
0–1	8·36	10·11	11·44
1–2	7·20	8·31	7·29
2–3	6·87	7·50	6·71
3–4	7·26	7·59	6·32
4–5	6·33	7·42	6·89
5–6	6·12	6·81	6·62
6–12	4·96	5·90	5·59
12–18	5·02	5·77	5·87
18–24	4·64	5·34	5·02
24–30	5·07	5·35	4·77
30–36	5·28	5·19	4·62
36–42	4·31	4·93	3·95
42–48	5·48	4·85	4·03
48–54	5·59	4·68	3·86

TABLE IX

Rate of Respiration: N_1K_1, N_3K_1, Ninth Leaf; N_5K_1, N_5K_5, Sixth Leaf

Hours in darkness.	Control	Increasing N deficiency		N- and K-deficient
	N_1K_1.	N_3K_1.	N_5K_1.	N_5K_5.
0–1	4·42	3·18	2·90	2·83
1–2	4·33	3·16	2·79	2·64
2–3	4·77	3·00	3·00	2·66
3–4	4·55	3·12	2·88	2·82
4–5	4·37	3·27	2·80	2·66
5–6	4·20	3·16	2·69	2·65
6–12	3·58	2·55	2·31	2·30
12–20	3·06	2·33	2·03	2·19
20–24	3·58	2·39	2·77	2·86
24–30	3·05	2·26	2·59	2·49
30–34	3·30	2·22	2·28	2·62
34–42	2·93	2·42	2·56	2·68
42–48	3·49	2·31	2·88	2·85
48–54	3·20	2·26	2·64	2·85
54–60	3·40	2·49	2·61	2·71
60–68	3·14	2·10	2·59	2·73

With potassium-starved leaves the most deficient series N_1K_5 has for the first hour the highest respiration rate, but the decline is here most rapid, so that after twenty-four hours the values are now the lowest. The control series has at first the lowest values, but towards the end of the experiment the values are highest. For the first forty hours, with the exception of the first reading, the intermediate series N_1K_3 give the highest respiration rates; later, however, they fall below the N_1K_1 values. In the nitrogen-starved series (Table IX), the respiration rate falls very much less. In the control series and the series N_3K_1 little change occurs after the first twenty hours, while in the completely starved series N_5K_1, N_5K_5, respiration remains nearly constant throughout the whole period; minor fluctuations do occur, but there is no general trend after the first six hours.

Table X

Reducing Sugar (mg. Hexose per gm. dry or fresh wt.)

	Control		Increasing K deficiency			
Leaf	N_1K_1.		N_1K_3.		N_1K_5.	
No.	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.
3	3·32	18·6	2·68	16·0	2·07	15·9
4	1·93	11·2	3·43	14·8	1·82	15·4
5	1·71	10·1	1·70	11·1	1·44	13·0
6	1·55	9·6	1·42	10·5	1·32	13·8
7	1·54	9·4	1·16	9·5	1·10	10·9
8	7·11	38·0	1·36	11·5	1·30	12·3
9	6·57	31·3	2·09	14·3	1·54	12·8
10	8·29	33·1	7·17	38·6	2·10	15·6
11	10·6	35·9	—	—	—	—
Mean	4·73	21·9	2·63	15·8	1·59	13·7

Table XI

Reducing Sugar (mg. Hexose per gm. dry or fresh wt.)

	Increasing N deficiency				N- and K-deficient	
Leaf	N_3K_1.		N_5K_1.		N_5K_5.	
No.	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.
3	2·76	16·3	2·79	17·5	2·65	16·4
4	3·53	18·5	3·81	21·9	3·57	21·3
5	1·55	12·8	2·58	12·4	2·44	11·2
6	2·52	11·3	2·54	10·6	2·22	8·8
7	1·98	7·6	2·22	8·6	2·36	9·4
8	2·02	7·3	2·82	10·6	2·89	10·8
9	2·94	10·8	3·15	11·7	3·31	12·5
10	3·39	12·6	—	—	—	—
Mean	2·59	12·1	2·82	13·3	2·78	12·9

Sugar content of the leaves.

The sugar contents of successive leaves are entered in Tables X and XI. The values for free reducing sugar are given in Tables X and XI on the basis of mg. equivalent of hexose per gm. fresh weight and per gm. dry weight respectively, and are shown graphically in Fig. 6*c*.

The values in Tables X and XI show the following relations: (1) In the control series and in the K-deficient series a minimum content of reducing sugar is reached in the seventh leaf, after which a very rapid increase is seen in the control series and to a lesser extent in the series N_1K_3 and N_1K_5. In the

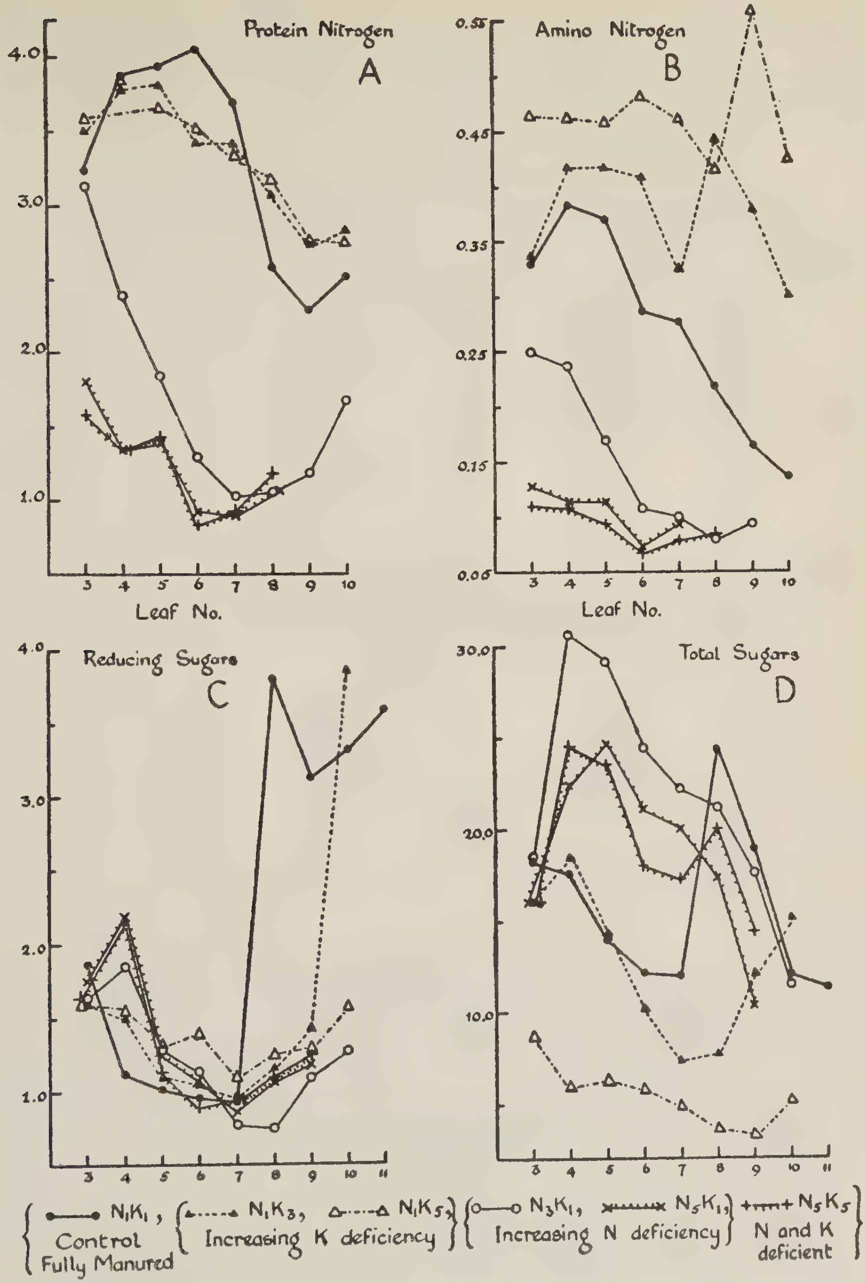

FIG. 6. Carbohydrate and nitrogen contents of successive leaves at emergence, all as percentage of dry weight. N_1 full nitrogen, N_3 $\frac{1}{9}$ nitrogen, N_5 $\frac{1}{81}$ nitrogen, K_1 full potassium, K_3 $\frac{1}{9}$ potassium, K_5 $\frac{1}{81}$ potassium.

nitrogen-deficient series the position of the minimum is not so sharply defined. On the fresh-weight basis it occurs in the fifth leaf, seventh leaf, and sixth leaf in the three series N_3K_1, N_5K_1, N_5K_5. Gregory and Baptiste (1936) have already reported a minimum at the fifth leaf in a N-deficient series comparable to the N_3K_1 and grown alongside the plants used in the present experiment. They also found a minimum in the seventh leaf of the control and K-deficient series. The very rapid increase in reducing sugar seen in the control, again confirms a similar finding for the 1932 series of plants reported by Gregory and Baptiste, though the sudden increase occurred in the ninth leaf, not in the eighth as here found. (2) K deficiency progressively lowers the reducing sugar content, while N deficiency has less effect when carried to levels of extreme starvation. In the earlier leaves reducing sugar is often higher in the N-deficient series than in the control, but the rapid increase after the seventh leaf does not here occur so that the mean values for the N-deficient series are lower than for the fully manured plants.

TABLE XII

Total Sugar as Equivalent Hexose (mg. per gm. dry or fresh wt.)

	Control		Increasing K deficiency			
Leaf	N_1K_1.		N_1K_3.		N_1K_5.	
No.	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.
3	32·6	181·7	26·8	160·0	11·3	86·7
4	30·3	176·9	29·3	185·4	7·0	59·3
5	23·4	138·9	22·0	143·3	7·1	64·2
6	19·6	121·3	13·9	102·4	5·6	58·2
7	19·5	118·7	9·0	73·9	4·8	47·6
8	45·6	243·5	9·1	77·0	3·9	36·4
9	39·8	189·3	17·7	121·1	3·9	32·6
10	30·2	120·4	28·2	151·9	7·0	51·8
11	33·2	112·7	—	—	—	—
Mean	30·5	155·9	19·5	126·9	6·3	54·6

TABLE XIII

Total Sugar as Equivalent Hexose (mg. per gm. dry or fresh wt.)

	Increasing N deficiency				N- and K-deficient	
Leaf	N_3K_1.		N_5K_1.		N_5K_5.	
No.	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.
3	31·4	185·0	25·7	161·4	25·8	160·1
4	58·4	306·2	39·2	225·2	41·3	246·3
5	57·8	291·4	51·2	247·3	51·6	236·1
6	54·3	243·7	50·6	211·3	45·0	179·1
7	57·9	221·6	51·6	200·3	43·3	172·6
8	58·9	211·9	46·3	173·7	53·5	200·0
9	47·9	175·9	27·8	103·3	38·2	144·0
10	30·1	111·5	—	—	—	—
Mean	49·6	218·4	41·8	188·9	42·7	191·2

In Tables XII and XIII, and graphically in Fig. 6D, are presented the values of total sugar content in successive leaves for all manurial treatments. The sugar values refer to total reducing substance in the alcohol extract after lead clearing and acid hydrolysis. It therefore includes some fructosan as well as small quantities of unidentified reducing substances. The values in the tables indicate the following conclusion: (1) Increasing K deficiency progressively reduces very greatly the total sugar content; nitrogen deficiency, on the contrary, very considerably increases the soluble carbohydrate. Even with extreme nitrogen deficiency, both with or without potassium deficiency, the mean sugar content is greatly increased, but the specific effect of potassium deficiency is completely masked by nitrogen deficiency as the good agreement between the series N_5K_1 and N_5K_5 shows. (2) In the control and K-deficient series the total sugar falls from the third leaf, reaching a minimum at the seventh (N_1K_1, N_1K_3) or eighth leaf (N_1K_5). After this a rapid rise occurs in the control series and a slower increase in the other series also, the rate of increase depending on the K level. The N-deficient series show a maximum sugar content in the seventh to eighth leaf with a fall in the last leaf. In all respects the present results agree with those for 1932 already published by Gregory and Baptiste (1936).

Protein and amino-acid content of the leaves.

The protein-N contents of the successive leaves are presented in Table XIV on the basis of percentage of dry weight, while the corresponding values for total amino N are given in Table XV. These values are graphically presented in Figs. 6A and 6B.

TABLE XIV

Protein-N Content (Percentage of dry wt.)

Leaf No.	Control N_1K_1.	Increasing K deficiency N_1K_3.	N_1K_5.	Increasing N deficiency N_3K_1.	N_5K_1.	N- and K-deficient N_5K_5.
3	3·23	3·50	3·58	3·13	1·81	1·58
4	3·87	3·78	3·79	2·39	1·34	1·35
5	3·94	3·81	3·66	1·84	1·39	1·42
6	4·06	3·42	3·52	1·29	0·93	0·83
7	3·70	3·42	3·35	1·02	0·90	0·92
8	2·58	3·08	3·18	1·06	1·06	1·19
9	2·29	2·73	2·76	1·18	—	—
10	2·52	2·84	2·75	1·69	—	—
Mean	3·27	3·32	3·32	1·70	1·24	1·21

The values in Table XIV show that the protein-N content of the fully manured series (N_1K_1) rises to a maximum at the sixth leaf and then declines, showing a recovery in the tenth leaf. The potassium-deficient series (N_1K_3, N_1K_5) closely resemble the control series, the maximum reached, however, being progressively advanced and of less magnitude. The minimum, on the other hand, is not so low in the K-deficient series and the later values are thus higher, so that the *mean protein content of the leaves is scarcely affected by potassium deficiency.*

The nitrogen-deficient series show a progressive reduction in protein content as the nitrogen level falls. The third leaf of the N_3K_1 series shows approximately the same value as the corresponding leaf of the control series, but with successive leaves the protein content declines more rapidly. In the completely starved series, even the third leaf already shows a much lower content of protein and a similar decline in the leaf succession is noted. The effect of K deficiency is again completely masked by N deficiency in the N_5K_5 series. The relations here found agree in every respect with the findings of Richards and Templeman (1936), and have been extended further to higher degrees of starvation.

TABLE XV

Total Amino-N Content (Percentage of dry wt.)

Leaf No.	Control N_1K_1.	Increasing K deficiency N_1K_3.	Increasing K deficiency N_1K_5.	Increasing N deficiency N_3K_1.	Increasing N deficiency N_5K_1.	N and K deficiency N_5K_5.
3	0·341	0·338	0·466	0·249	0·126	0·110
4	0·383	0·417	0·464	0·238	0·113	0·108
5	0·371	0·418	0·437	0·169	0·112	0·095
6	0·288	0·409	0·482	0·108	0·073	0·069
7	0·278	0·326	0·464	0·099	0·096	0·079
8	0·218	0·444	0·415	0·080	—	0·086
9	0·165	0·380	0·559	0·096	—	—
10	0·138	0·302	0·427	—	—	—
Mean	0·273	0·379	0·464	0·148	0·104	0·091

The effects of K deficiency on total amino N stand in marked contrast with the effect on protein N. With increasing K deficiency the amino-N content is progressively *increased*, and this effect is particularly clear with the later leaves. In the control series the amino-N content steadily declines with leaf succession after an ill-defined maximum at the fourth leaf. In the N_1K_3 series with moderate K deficiency a higher maximum appears at the fifth leaf and the decline is less marked, while in the completely starved series N_1K_5 the maximum appears at the sixth followed by a still higher value in the ninth leaf; there is here no progressive fall in amino N. The mean values show an increasing amino-N content with progressive K starvation.

In the N-deficient series the amino nitrogen presents a very similar picture to the protein N as all N fractions are reduced as N starvation increases. No effect of K deficiency is seen in the N_5K_5 series, which closely resemble the N_5K_1 series. These results again confirm those of Richards and Templeman (1936).

STATISTICAL ANALYSIS OF EXPERIMENTAL RESULTS

The relation of the respiration rate to the levels of the carbohydrate and nitrogen fractions studied has been investigated by the method of correlation. The mean values of respiration rate and of the variables studied differ greatly in the different series as may be seen from the summary in Table XVI, which brings together the relevant data. The second column gives the numbers of leaves in the succession used for determining the mean values of the variables. As analyses of all leaves were not made the number of serial leaves used varies with the different treatments and the mean values are therefore not always identical with those stated earlier in Tables V–XV, but they include all the data on which the statistical examination was performed.

TABLE XVI

Mean Values of Respiration Rate and Analytical Data for the Various Manurial Series. (Respiration rate in mg. per gm. dry wt. per hr.; other data as percentage of dry weight.)

Treatment.		Leaves included.	Respiration (*R*).	Free reducing sugar (*s*).	Sucrose (*S*).	Protein N (*P*).	Amino N (*A*).
Control	N_1K_1	3–10	6·00	2·02	14·13	3·27	0·273
K-deficient	N_1K_3	3–10	7·86	1·58	11·11	3·32	0·379
	N_1K_5	3–10	7·46	1·37	4·10	3·32	0·464
N-deficient	N_3K_1	3–9	4·49	1·21	22·14	1·70	0·148
	N_5K_1	3–7	4·13	1·38	19·02	1·25	0·098
	N_5K_5						

TABLE XVII

Correlation Coefficients between the Factors Respiration (R), Reducing Sugar (s), Sucrose (S), Protein N (P), Amino N (A)

			5 % point.	1 % point.
sP +0·053	*sA* −0·059	*Rs* −0·057	0·309	0·398
		Rs.AP −0·126	0·317	0·408
SP −0·614	*SA* −0·751	*PA* +0·862	0·309	0·398
SP.A +0·099	*SA.P* −0·554	*PA.S* +0·769	0·313	0·403
SP.R −0·431	*SA.R* −0·704	*PA.R* +0·549	0·313	0·403
SP.RA −0·074	*SA.PR* −0·197	*PA.RS* +0·382	0·317	0·408
			5 % point.	1 % point.
RP +0·843	*RA* +0·824	*RS* −0·488	0·309	0·398
RP.A +0·464	*RA.P* +0·355	*RS.P* +0·069	0·313	0·403
RP.AS +0·460	*RA.PS* +0·409	*RS.PA* +0·341	0·317	0·408
RP.ASs +0·451	*RA.PSs* +0·431	*RS.PAs* +0·365	0·321	0·413

A cursory examination of the table discloses a marked contrast between the two deficiency series. Whereas the high respiration rate in the potassium-deficient series is associated with a very low content of carbohydrate, in the nitrogen-deficient series low respiration is associated with a very high content of carbohydrate. Again the relation of respiration to protein N and amino N is the reverse of that to carbohydrate in the two series. To investigate the relations of the factors all available data were first used, giving in all a total of forty-one sets of values for the statistical analyses. The values of the correlation coefficients are given above (Table XVII), together with the requisite values for $P = 0·05$ and 0·01.

The total correlations between respiration rate and the levels of the internal factors show high positive values for protein N and amino N, and negative values for sucrose and reducing sugar, all of which except the last are highly significant. These correlations, however, undergo marked changes when the effects of the other factors are eliminated, the partial correlation with the nitrogen fractions falling, while that in the sucrose changes sign; the significance for both nitrogen fractions, as shown by the values of Z, being greater than 100:1, and that for sucrose greater than 20:1. The partial correlation with reducing sugar, however, remains quite without significance. Undoubtedly, therefore, respiration rate is related to the factors, protein N, amino N and sucrose, with each of which it is positively correlated, while free reducing sugar is without effect. This generalization is based on a wide range of the variables and gives the average effects of the factors. The conclusion requires modification when the separate series are considered, and it will then be seen that the low values of the partial correlations is due to the opposite relations holding in the nitrogen- and potassium-deficient series.

The correlations between the nitrogen and carbohydrate fractions are not without interest. The total correlations of sucrose with protein N and amino N are very significantly negative, but when the effects of respiration are eliminated in the former case the correlation is reduced, while in the latter it is scarcely affected. When the effect of amino N is eliminated the correlation of sucrose with protein almost vanishes, and that in the amino N is considerably reduced when the effect of protein N is eliminated. In either case, when both the effect of respiration and the nitrogen fraction is eliminated the correlations become quite insignificant. It would appear, therefore, that the relative levels of the carbohydrate and nitrogen fractions is determined to a large extent by the respiration. The high positive correlation of protein N to amino N falls as the effects of the other factors are eliminated but remains positive and highly significant.

The extent to which variation in respiration rate, using all the data available, is dependent on variation in the levels of the factors studied is shown by the multiple regression coefficient, which has the value $R = 0{\cdot}8834$, showing that 78 per cent. of the total variance is accounted for. The corresponding value of Z for variance due to regression and deviations from regression is $Z = 1{\cdot}7329$ (1 per cent. point $= 0{\cdot}6954$). The regression equation is:

$$R = -0{\cdot}166s + 0{\cdot}0762S + 0{\cdot}9918P + 7{\cdot}9373A + 0{\cdot}3757,$$

the variables being all stated as percentage of the dry weight, and respiration as mg. CO_2 per hour per gm. dry weight. The equation shows the change in respiration for a change of 1 per cent. in the dry weight for each of the factors studied: the preponderating effect of the nitrogen fractions is evident.

Within the manurial series the relations of the variables are shown by the correlation coefficients entered in the tables below. The values of the correlation coefficients for pairs of single factors are presented in Table XVIII, together with the values for $P = 0·05$ and $0·01$ respectively. Significant values are indicated. Since the values of the N_5K_1, N_5K_5 series scarcely differ they have been treated as a single series.

TABLE XVIII

Correlation Coefficients for Pairs of Single Factors

Treatment.	Respiration rate (*R*).				Free reducing sugar (*s*).			Sucrose (*S*).		Protein N (*P*).
	s.	*S.*	*P.*	*A.*	*S.*	*P.*	*A.*	*P.*	*A.*	Amino N (*A*)
N_1K_1	−0·689	+0·194	*+0·724*	**+0·962**	+0·035	**−0·953**	*−0·789*	−0·264	+0·174	**+0·836**
N_1K_3	−0·143	*+0·800*	**+0·838**	+0·286	+0·197	−0·481	−0·612	+0·469	+0·043	+0·328
N_1K_5	+0·289	**+0·864**	+0·494	−0·344	+0·533	+0·138	−0·111	+0·680	−0·234	−0·245
N_3K_1	**+0·879**	−0·039	**+0·984**	**+0·942**	+0·339	**+0·879**	**+0·936**	+0·071	+0·274	**+0·969**
N_5K_1 N_5K_5	+0·434	−0·368	**+0·834**	*+0·731*	+0·063	*+0·667*	*+0·730*	−0·109	+0·008	**+0·883**

N_1K_1, N_1K_3, N_1K_5: 5% point = 0·707 } N_3K_1 5% point = 0·754 } N_5K_1 5% point = 0·631
1% „ = 0·834 } 1% „ = 0·875 } N_5K_5 1% „ % 0·765

The following relations between the factors affecting respiration rate may be noted.

Fully manured series (N_1K_1). There is high, but not significant, negative correlation with free reducing sugar; quite insignificant correlation with sucrose; high and significant positive correlation with protein N, rising to even higher value in the amino N.

Nitrogen-deficient series (N_3K_1, N_5K_1, N_5K_5). Free reducing sugar is positively correlated with respiration, reaching in the medium deficient series N_3K_1 a very high positive value; with sucrose on the contrary insignificant negative values obtain. With nitrogen fractions in all cases highly significant positive correlations appear.

Potassium-deficient series (N_1K_3, N_1K_5). The correlations with free reducing sugar are quite insignificant; with sucrose highly significant positive values are found, which increase in magnitude as the deficiency is raised. With protein N a highly significant value is obtained in the intermediate series N_1K_3, while with amino N there is no significant correlation.

The correlations between the levels of the various nitrogen and carbohydrate fractions are also given. In the fully manured series significant *negative* correlations are found between reducing sugar and both the protein and amino N, while in the nitrogen-deficient series significant *positive* values of this correlation appear. Sucrose, on the other hand, in all series shows only insignificant correlations with reducing sugar, although all are positive. With both protein N and amino N correlations with sucrose are not significant in value, and in the N_1K_5 series alone does the positive value found with protein N at all approach significant level. In the fully manured and the nitrogen-deficient series protein and amino N levels are highly positively correlated, whereas with K deficiency no consistent or significant relation appears. This confirms the relations found by Richards and Templeman (1936) between the nitrogen fractions in that nitrogen deficiency reduces the general level but not the relative amounts of protein and amino N, while with K deficiency the amino N is relatively greatly increased.

Partial correlation coefficients were also calculated, but for reasons of space are not given in full. The significant correlation with respiration rate obtained in the different series are alone entered in Table XIX.

TABLE XIX

Partial Correlation Coefficients with Respiration Rate in all Manurial Series: Significant Values only Entered

Partial correlation coefficient.	K-deficient series N_1K_5.	N_1K_3.	Fully manured N_1K_1.	N-deficient series N_3K_1.	N_5K_1 N_5K_5.
Rs.S	—	—	—	+0·949	—
RS.P	+0·829	+0·844	—	—	—
RS.A	+0·859	+0·824	—	−0·920	—
RS.s	+0·877	+0·854	—	—	—
RP.A	—	+0·829	—	—	—
RP.S	—	+0·874	+0·819	+0·990	+0·859
RP.s	—	+0·886	—	+0·928	+0·812
RA.P	—	—	+0·942	—	—
RA.S	—	—	+0·961	+0·990	+0·789
RA.s	—	—	+0·945	—	—

N_1K_1, N_1K_8, N_1K_5: 5 % point = 0·755, 1 % „ = 0·875; N_3K_1 5 % point = 0·878, 1 % „ = 0·959; N_5K_1 5 % point = 0·666, N_5K_5 1 % „ = 0·798

In general the values of the partial correlations confirm those of the total correlations in Table XVIII. In the fully manured series the high positive correlation of respiration rate with amino N remains after eliminating the effects of each of the other factors, but with protein N the significant correlation vanishes when the effect of amino N is eliminated. In the nitrogen-deficient series the significant positive correlation with protein N does not hold when the effect of amino N is eliminated, and with amino N significant correlation vanishes when the effect of protein N or reducing sugar is eliminated. In the N_3K_1 series significant correlation with free reducing sugar vanishes when the effect of amino N or protein N is eliminated, while with sucrose in this series a highly significant negative correlation in the respiration appears. The agreement between the two N-deficient series is very striking with respect to the relation of respiration and nitrogen level. In the potassium-deficient series the same significant partial as total correlations are found. In both series significant positive correlation with sucrose holds, and in the intermediate series N_1K_3 with protein also.

Variations in the relative importance of nitrogen level and carbohydrate level in the different series is thus apparent. To establish this relation as clearly as possible the correlations of respiration with the levels of the nitrogen and carbohydrate fractions respectively, when the effects of both components of the other are eliminated, have been calculated and are presented in Table XX. Owing to the few degrees of freedom available the requisite level for significance is very high, but in spite of this significant values are found. Again the same relations of the factors appear: in the potassium-deficient series N_1K_5 sucrose alone shows significant correlation, the nitrogen fractions are almost without effect.

In the potassium-deficient series N_1K_3 significant correlation obtains between respiration and sucrose as well as protein N. In the fully manured

TABLE XX

Partial Correlations of Respiration with Carbohydrate Fractions, Eliminating Effect of both Nitrogen Fractions and vice versa

Partial correlation coefficients.	K-deficient series N_1K_5.	N_1K_3.	Fully manured N_1K_1.	N-deficient series N_3K_1.	$\underbrace{N_5K_1\ N_5K_5}$.
RS.PA	*+0·838*	*+0·865*	−0·548	−0·735	−0·514
Rs.PA	+0·245	+0·681	+0·042	+0·568	−0·317
RP.Ss	−0·467	*+0·829*	+0·792	*+0·961*	*+0·822*
RA.Ss	−0·310	+0·339	*+0·931*	+0·930	*+0·721*

N_1K_1, N_1K_3, N_1K_5: 5 % point = 0·811; 1 % „ %0·917 | N_3K_1 5 % point = 0·950; 1 % „ = 0·990 | N_5K_1 5 % point = 0·707; N_5K_5 1 % „ = 0·834

series N_1K_1 significant correlation with amino N alone is found, though with protein N the high positive value is very near the significant level. The nitrogen-deficient series N_3K_1, N_5K_1, N_5K_5, all show highly significant positive values with protein N, and high values in the amino N also, though only one of these is significant. In the nitrogen-deficient series negative correlations are found with sucrose, but fail to reach significant level. In none of the manurial series is respiration significantly correlated with free reducing sugar.

The multiple regression coefficients and the regression equations for all series are given below:

	Multiple regression coefficient.	
N_1K_1	0·956	$R = -0{\cdot}1510s + 0{\cdot}0563S - 0{\cdot}4877P + 24{\cdot}03A + 0{\cdot}549$
N_1K_3	0·964	$R = +0{\cdot}2496s + 0{\cdot}1527S + 2{\cdot}073P + 4{\cdot}110A - 2{\cdot}676$
N_1K_5	0·876	$R = -1{\cdot}644s + 0{\cdot}8024S + 0{\cdot}2372P + 2{\cdot}379A + 4{\cdot}530$
N_3K_1	0·984	$R = -0{\cdot}1695s - 0{\cdot}0058S + 1{\cdot}971P - 0{\cdot}8262A + 1{\cdot}594$
$\left.\begin{array}{l}N_5K_1\\N_5K_5\end{array}\right\}$	0·850	$R = -0{\cdot}5371s + 0{\cdot}0684S + 2{\cdot}852P + 2{\cdot}364A - 0{\cdot}222$

The statistical analysis thus confirms the contrast in the effects of nitrogen and potassium deficiencies. In the former case the levels of protein N and amino N, in the latter the level of sucrose, are the predominant factors in determining respiration rate. The fully manured series occupies an intermediate position and resembles the nitrogen-deficient series rather than the potassium deficient.

The multiple regression coefficients show that even in the least satisfactory case (N_5) 72 per cent., in the most satisfactory (N_3) 97 per cent., of the variability in respiration is accounted for by variation in the factors studied.

GENERAL DISCUSSION

The relation of respiration rate to the range in variation in contents of carbohydrate and nitrogen is shown graphically in Figs. 7–9. Dealing first

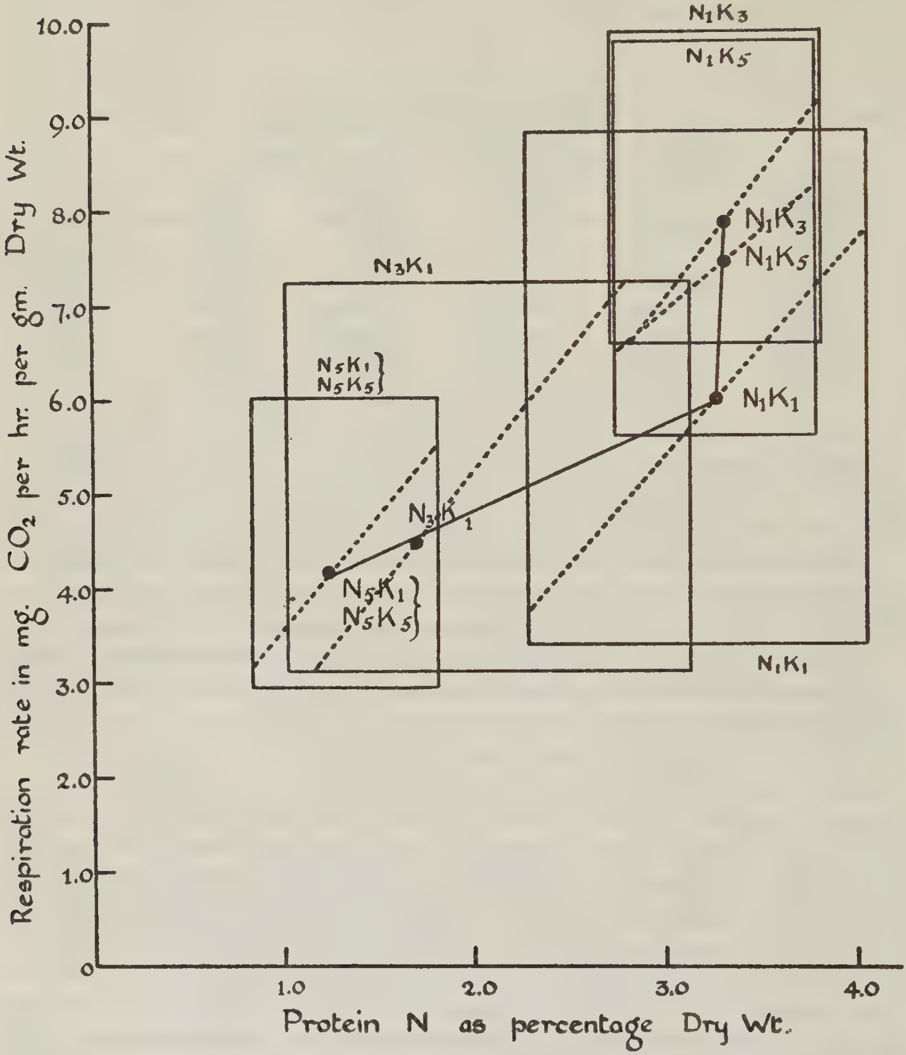

FIG. 7. Relation of respiration rate to protein content in the various manurial series. The rectangles enclose the values for the successive leaves in each manurial series. The regression of respiration on protein N is shown as a dotted line for each manurial series. The mean values for each series are marked on the regression lines. For further details see text.

with the data of protein in Fig. 7; along the abscissae are plotted protein content of the leaves (per cent. dry weight), and along the ordinate the respiration rate (mg. CO_2 per hr. per gm. dry wt.). The rectangles are drawn to enclose all the data belonging to a particular manurial series. The individual values for the successive leaves are not plotted, but instead the regression lines of respiration rate on protein content are drawn, and on these lines the mean values of protein content and respiration appropriate to each manurial series are marked. The rectangles for the series of increasing nitrogen (N_5K_1, N_3K_1, N_1K_1) partially overlap; the higher the nitrogen the higher the protein content and the greater the extension of the rectangle in the direction of higher respiration. The mean values of respiration in this series lie on a straight line, showing increasing respiration with increasing protein content. The rectangles corresponding with the potassium-deficient series (N_1K_3, N_1K_5) lie completely within the range of the fully manured series so far as protein content is concerned. The mean values of protein in this series lie vertically above those of the fully manured series, indicating that increasing potassium deficiency leads to increase in respiration rate with constant protein content. The regression lines for the various treatments do not differ significantly in slope, showing that at all protein levels, the effect of variation in this factor on respiration rate is constant within the limits of error of this experiment. The variation on level of the regression lines as between different treatments must therefore be due to effects of factors other than protein content.

From the figures in Table XVI the relations between respiration rate and mean protein content for each manurial series has been calculated, and are as follows: N_1K_1 0·184, N_1K_3 0·237, N_1K_5 0·225; N_3K_1 0·264, $\left.\begin{matrix}N_5K_1\\N_5K_5\end{matrix}\right\}$ 0·330. The figures represent gm. CO_2 respired per hour per gm. protein N content; per gm. protein the values will be approximately one-sixth as great. It is noteworthy that respiration calculated on the basis of protein-N content shows a minimum value in the fully manured plants, and is higher in all deficient series. Incidentally, these figures show that protein N is not a satisfactory basis on which to compare respiration rate of plants variously manured.

In Fig. 8 the same method of graphical representation is applied to the data for amino N. The series of increasing nitrogen content show rectangles extending in the direction of increasing amino N, and also in the direction of increasing respiration. The potassium-starved series however now show a further extension in both directions beyond the range of the fully manured series. The minimum level of respiration shows a marked increase in the N_1K_3 series. The mean values of respiration for all series lie on a smooth curve rising in the direction of increasing amino acid with a well-marked optimum at the K_3 level. The regression lines in the nitrogen-deficient series differ little in slope from the fully manured, but with increasing potassium deficiency the slopes of the regression lines change abruptly, the slope being reversed in the N_1K_5 series. These results are implicit in the correlation coefficients already presented. That the respiration rate shows a maximum with mean level of amino N is elucidated by a consideration of the relation to sugar content. As the partial correlation coefficients had in no series shown a significant relation of respiration to reducing sugar (Table XX) the values for sucrose are alone presented in Fig. 9. The method of presentation is similar to that already used for the nitrogen fractions.

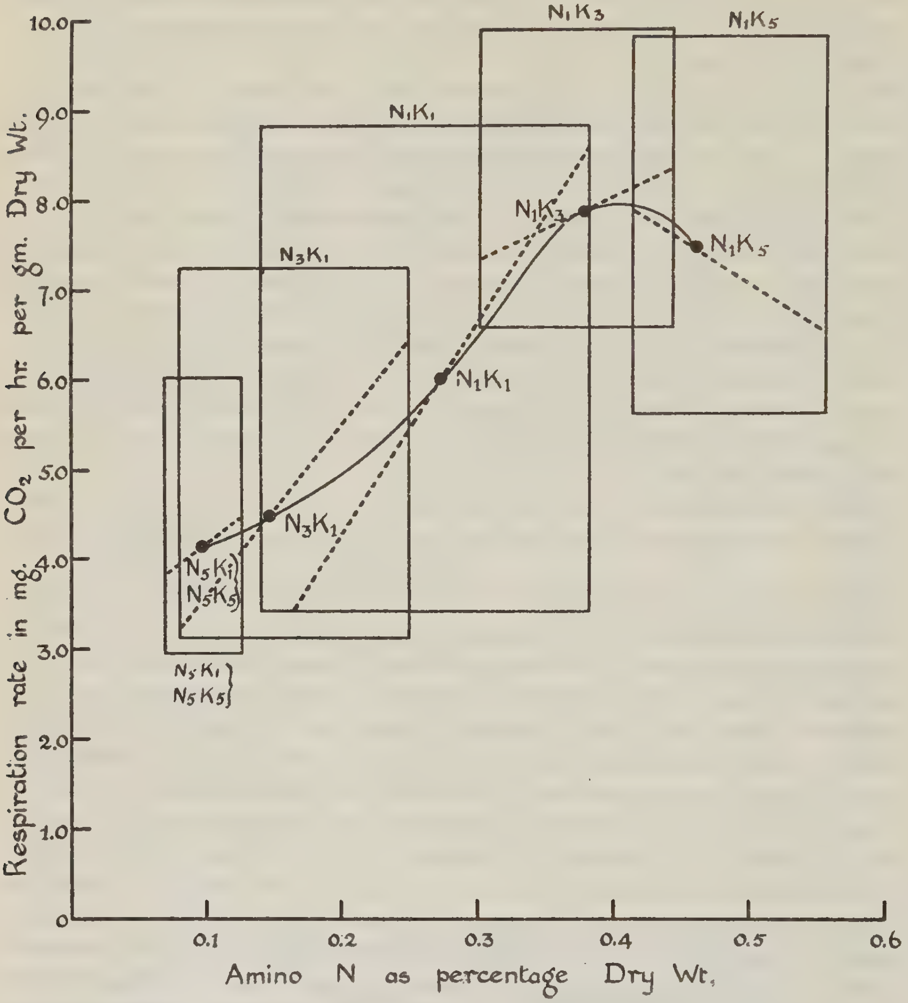

FIG. 8. Relation of respiration rate to amino-acid content in the various manurial series. The rectangles enclose the values for the successive leaves in each manurial series. The regression of respiration on amino N is shown as a dotted line for each manurial series. The mean values for each series are marked on the regression lines. For further details see text.

The fall in level of sucrose with potassium deficiency and rise with nitrogen deficiency is clearly shown. The regression lines show maximum slope in the N_1K_5 series, and decreasing slope as potassium is increased. With deficiency of nitrogen the relation of respiration to sucrose content becomes

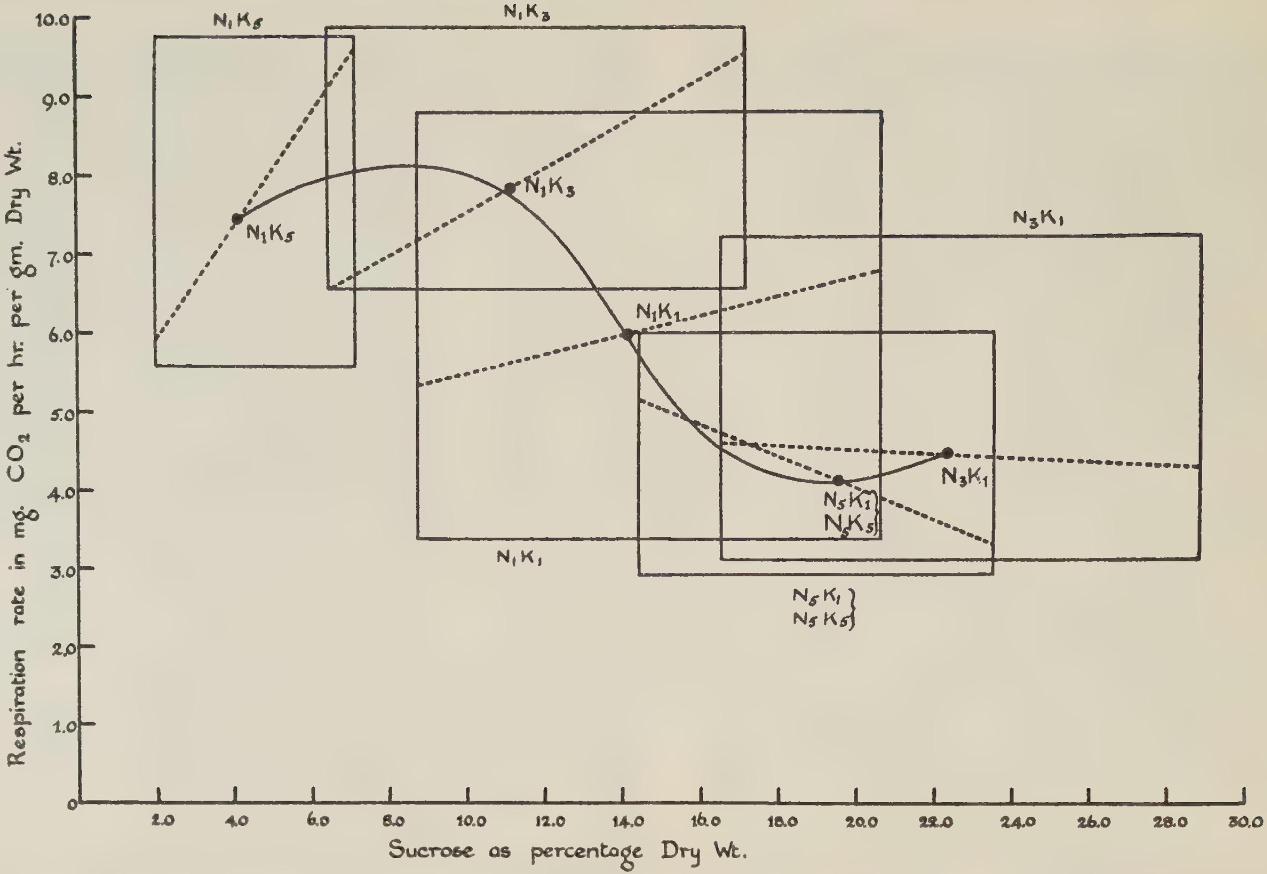

FIG. 9. Relation of respiration rate to sucrose content in the various manurial series. The rectangles enclose the values for the successive leaves in each manurial series. The regression of respiration on sucrose is shown as a dotted line for each manurial series. The mean values for each series are marked on the regression lines. For further details see text.

negative (cf. Table XVIII). Only in the case of the potassium-deficient series is there a significant positive correlation with sucrose (Tables XIX, XX).

Reviewing the results as a whole it is clear that in the nitrogen-deficient series the effects of amino acid and protein cannot be separated. As the values in Table XVIII show the correlation between amino N and protein N is so high in the fully manured and nitrogen-deficient series, that when the effect of either is eliminated, the correlation of the other with respiration vanishes (Table XIX). In the potassium-deficient series, on the other hand, the values in Table XVIII show no close correlation between amino N and protein N, so that it is here possible to test whether respiration is more closely related to protein N or amino N content. Dealing with the fully manured and potassium-deficient series together, since the range of amino-N variation within the individual deficient series is small (Table XV) and also to increase the degrees of freedom available for estimation of correlation, the following data were calculated. The correlations of respiration with protein N and amino N respectively, eliminating the effect of the carbohydrate fractions and of the other nitrogen fraction, gave the following values of partial correlation coefficients:

$$\left.\begin{array}{l} RP.ASs = 0{\cdot}4072 \\ RA.PSs = 0{\cdot}6840 \end{array}\right\} \begin{array}{l} 5\% \text{ point } 0{\cdot}4329 \\ 1\% \text{ point } 0{\cdot}5487. \end{array}$$

It appears then that in the potassium-deficient series the amino-N content is very definitely correlated with respiration, whereas with protein N there is no significant correlation. This of course merely confirms the relations seen in Fig. 7, in which mean protein remains constant in the potassium-deficient series, while the amino N rises with deficiency and is accompanied by increased respiration.

The fall in respiration rate from the N_1K_3 to N_1K_5 series is due to the effect of sucrose. In the series N_1K_5 the sugar level is much lower than in the N_1K_3 and respiration rates within the former series (successive leaves) is almost completely determined by variation in sucrose. The dependence of respiration on the level of potassium manuring is thus determined by two opposed relationships: (1) with decrease in potassium supply respiration rises, (2) with extreme deficiency this increase is opposed by the low level of sugar found in the leaves. The interaction of these processes results in an optimum respiration at medium potassium deficiency. These experiments thus confirm those of Richards (1932) and show that his suggested explanation is correct.

It is clearly necessary to bring the experimental results above into some relationship with current views on the respiratory process, and to endeavour, however tentatively, to discuss the interrelation of carbohydrate and nitrogen metabolism in determining the levels of respiration found in the successive leaves within a single manurial series, as well as the differences in respiration level between the various series. That these relations are complex needs no stressing. The respiration of carbohydrate and the synthesis of protein each represents a problem which is as yet a matter of conflicting views, and moreover these have been studied rather as isolated fields than as part of a functional synthesis. It has been necessary to lean somewhat heavily on the views expressed by specialists in these fields, and to this end the reviews of Robinson (1929), Onslow (1931), McKee (1937), and Nightingale (1937) for protein synthesis, and of Onslow (1931), Bennet-Clark (1933), and Turner (1937) for respiration in relation to carbohydrate metabolism, have been consulted. To provide a basis for this discussion the following schema is tentatively presented.

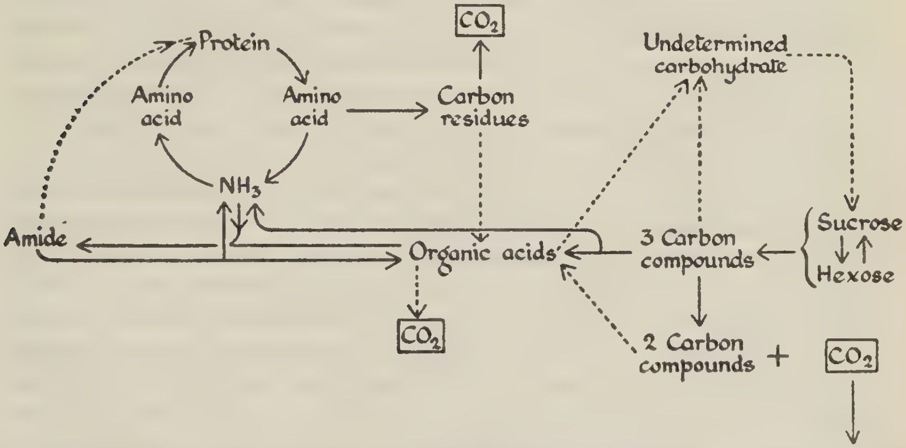

FIG. 10. A schema of relations of the carbohydrate and protein metabolic cycles indicating possible origin of CO_2.

So far as protein synthesis is concerned the main problems appear to be, (1) the immediate precursor of protein whether amino acid or amide, (2) the relation of synthesis to proteolysis. The older view that amide plays a special role in protein synthesis is now out of favour on the grounds of the unlikelihood that protein synthesis should always proceed through asparagine or glutamine. The view has gained ground that amide synthesis is a means of removal of toxic ammonia, and is an intermediate storage product which may, however, be translocated to the seat of active protein synthesis. The course of synthesis from amide direct to protein has therefore been shown in the schema as a dotted line only. Amide then becomes important as a potential source of ammonia by deamination. The ultimate fate of the carbon residue will be considered later. It may be assumed, therefore, that protein synthesis always begins with ammonia, the precursors of protein being amino acids formed either from ammonium salts of saturated and unsaturated organic acids; or possibly direct from the 3 carbon intermediate products of glycolysis, by condensation *en bloc* to form large polypeptide complexes including heterocyclic rings (Onslow, 1931). An alternative link between the protein and carbohydrate cycles excluding organic acid has therefore been included in the schema.

The relation between synthesis and degradation of protein must be considered. Two alternative views are here possible, namely, (1) that protein synthesis is a reversal of proteolysis, the direction of the reaction being determined by mass action and the velocities of the reactions concerned; (2) that the two processes are quite distinct and are catalysed by separate systems of enzymes, the intermediate amino acids being possibly chemically different. In the former case the relative concentrations of protein and amino acid would tend to constant values, and an increase in amino N relative to protein N could only result from an increase in concentration of the stage previous to amino acid formation. In the latter case amino N may increase by break-down of protein irrespective of the concentration of the first products of protein synthesis. Only in this case can a real cycle of nitrogen metabolism be postulated, and this possibility is represented in the schema. Mothes (1933) has discussed the factors affecting proteolysis and stresses the importance of water, carbohydrate supply, and oxygen tension. He appears to favour the view of independence of the processes of synthesis and hydrolysis, the protein content at any time being determined by the velocities of the independent reactions, and regards both processes as continually at work.

The position of organic acids as possible precursors of protein synthesis is clearly important. Bennet-Clark has discussed the relations of organic acid and gives strong evidence for a carbohydrate origin. The suggested origin of organic acid by deamination of amino acid put forward by Ruhland and Wetzel (1927, 1929) for acid plants, which has been widely accepted subsequently, would appear to be much less certain, even in this special case, since the metabolism of the rhubarb has been reinvestigated by Bennet-Clark and Woodruff (1935). As oxidative deamination of certain of the amino acids would lead to succinic acid formation this possible source of organic acid is included in the schema. The origin of organic acids from either 3 C or 2 C compounds as shown in the diagram has followed the suggestion of Bennet-Clark, as also the direct anabolic formation of carbohydrate of indeterminate nature from organic acid. The oxidative anabolism of triose suggested by F. F. Blackman (1928) and recently discussed very fully by Turner (1937) is indicated. The schema thus shows metabolic cycles of carbohydrate and nitrogen linked through organic acid.

The question of the origin of the CO_2 of respiration now requires consideration. The main source of CO_2 is generally accepted to be free carbohydrate, and only in cases of carbohydrate starvation is CO_2 presumed to arise from protein break-down. A preferential oxidation of carbohydrate has been postulated, and according to McKee (1937) the respiratory quotient of detached leaves in the dark has been shown by Yemm to indicate protein respiration only under conditions of advanced starvation. Under anaerobic conditions there is general agreement that CO_2 originates from 3 C compounds after phosphorylation of carbohydrate, through pyruvic acid formation followed by decarboxylation. The aldehyde produced reacts with triose phosphate to produce phosphoglyceric acid and alcohol. From the phosphoglyceric acid pyruvic acid is reformed with liberation of phosphate. A cyclic process is thus established, the phosphate being continuously carried back to the carbohydrate through the intervention of the nitrogen compound adenosin. As to the production of CO_2 aerobically there is at present no general consensus of opinion. According to Boysen-Jensen and Lunsgaard the sugar breakdown in fermentation bears no relation to that in aerobic respiration. In oxygen the formation of 3 C compounds from sugar ('triosis', Turner (1937)) is prevented either by a reversible inhibition of the necessary enzymes or by preventing access of the substrate to the enzyme. On the other hand, Blackman has suggested that the products of triosis are in oxygen built back to some unknown carbohydrate. The question is discussed very fully by Turner (1937).

In the succulent plants Bennet-Clark has postulated the production of organic acid from sugar. For each molecule of sugar converted to acid two atoms of carbon are converted into some other compound, not CO_2 or other volatile carbon compound, and a building back to polysaccharide is suggested. With increased oxygen tension more acid is produced.

It may be suggested that possibly the condition in the succulent plant represents only an exaggerated version of a mechanism which always occurs in aerobic respiration, namely conversion of triose to organic acid, but that normally the organic acid is largely used for protein synthesis. In the succulent plants nitrogen metabolism is usually slow, as is seen from the slow growth and development of such plants. Nitrogen supply may well limit continuously the rate of protein metabolism, so that the intermediate organic acids accumulate. Low organic acid content in the tomato has been found in plants fed with ammonium as compared with nitrate by Clark (1936). Bennet-Clark has shown that in *Sedum praealtum* organic acid is reversibly converted quantitatively into sedoheptose, under the influence of light and dark, without CO_2 loss, and presumably, therefore, malic acid as such is not respired.

The production of CO_2 by oxidative deamination has been recognized as a possible source of CO_2 in the starving leaf. The keto acid thus formed is decarboxylated with production of aldehyde and CO_2. The fate of the carbon residue in this process is not known, and this problem is an exactly similar one to that of the fate of acetaldehyde in aerobic respiration. The suggestion of Ruhland and Wetzel that organic acid accumulation occurs in this way must be considered as unproven. It appears possible that complete oxidation to CO_2 takes place. The same may be true of succinic acid produced by deamination and deamidization of asparagine, in the utilization of amide. In this connexion the fact is significant that very high rates of CO_2 production are found in meristematic regions. Here amide or amino acid is available by translocation from the older parts. The adequacy of this movement of nitrogen is well shown by the barley plant which requires external nitrogen only for the first six weeks of growth, at which time the main part of the developmental cycle, namely flower production, is not yet initiated; in the complete absence of external sources of nitrogen further development is no way hindered. The meristem, moreover, contains no stored carbohydrate: the high CO_2 production may therefore to a considerable extent arise from carbon residues of amide and amino acid after deamination. In this view the main function of carbohydrate respiration would be to supply precursors of protein synthesis, and protein will be rebuilt always from fresh sources of carbon, previously used carbon being eliminated by respiration.

The effect of amino acid feeding on respiration rate (Spoehr and McGee (1923), Genevois (1927)) is also pertinent to this problem. Schwabe (1932) has shown that with *Elodea* respiration is increased 300 per cent. by feeding with tyrosine or glutamic acid, and further that after transference to water the increased respiration soon falls to normal, and the increased rate may be repeated at will. He concludes from estimates of respiratory quotient that amino acid is not directly respired away, but favours the view that it acts as hydrogen acceptor. It would appear that the respiratory quotient is not an unequivocal indicator of metabolism when many oxidations are simultaneously occurring. It may happen that the aggregate respiratory quotient approaches unity though large variations in the ratios of individual reactions may hold. Alternatively the increase in CO_2 production in Schwabe's experiments may be attributed to deamination and protein synthesis. The foregoing discussion has been intended to lead to the following tentative conclusion: the amount of CO_2 produced may reflect the rate of synthesis and break-down of protein in a cyclic process. Under anaerobic conditions CO_2 production by glycolysis and decarboxylation no doubt occurs, but under aerobic conditions metabolism of carbohydrate may be linked very closely with protein metabolism, the products of triosis being used for protein synthesis, and carbon dioxide may be eliminated at the protein break-down. In this connexion the reaction between amino acids and iodoacetic acid noted by Michaelis and Schubert (1934) may be of some importance. If such a protein cycle exists CO_2 production would reflect the rate at which the cycle is traversed. The experimental results will now be discussed in relation to the considerations put forward.

The data presented above show that the low respiration rate of the nitrogen starved leaf is associated with a high carbohydrate content. This may be accounted for by the four following hypotheses: (1) unavailability of the carbohydrate, (2) a lack of the enzymes concerned in glycolysis, (3) inhibition of these enzymes, (4) the low rate of protein synthesis and break-down.

In the leaves of barley there is little accumulation of starch, all the carbohydrate is present in soluble form, and therefore there should be no hindrance to contact of enzyme and substrate in the cell.

The experiments of Ruhland and Ullrich (1929) and Hamner (1936) have shown that large increases in respiration can result from feeding leaves with inorganic nitrogen, and in the work of Hamner this has been established for the nitrogen-starved plant. Work in this Institute has shown that feeding isolated barley leaves with ammonium nitrate and sugar leads to large increase in respiration rate (Sankaran, 1936). It appears, therefore, that either the necessary enzymes are already present or can be rapidly synthesized in the leaf if inorganic nitrogen is given. Inhibition by lack of phosphorus is unlikely as inorganic phosphate in the experiments here described is present in high concentration in the nitrogen-starved leaf.

The fourth hypothesis must therefore be examined. By feeding nitrogen-starved leaves at any stage of development of the plant a deep green colour is rapidly induced, and without much doubt a great increase occurs in all nitrogen fractions. The close relation between chlorophyll production and protein metabolism has recently been investigated by Michael (1935), who showed that feeding leaves with asparagine and sugar prevented yellowing and caused protein synthesis.

This chlorophyll and protein break-down in the dark was accelerated by high oxygen tension, and partially inhibited by low tension of oxygen (7 per cent.), indicating that oxidative deamination is concerned. The increased CO_2 production found in other feeding experiments is therefore in all probability associated with a protein metabolism, and this was in fact shown by Sankaran (1936) in the barley leaf. Further, in the experiments here reported, it has been seen that the falling respiration in the dark is also intimately associated with the level of nitrogen metabolism (Tables VIII, IX). In the early leaves of the fully manured plants a rapid decline in respiration in the dark is seen, which in the later leaves no longer appears (Table VII). The later leaves resemble the nitrogen-starved leaves in showing a long-sustained, steady respiration rate.

A close parallelism holds between nitrogen metabolism and respiration in the successive leaves of the fully manured plants, which is moreover independent of the variation in carbohydrate supply. In the leaves of the nitrogen-starved plants the low respiration is associated with a low level of nitrogen, but by feeding with inorganic nitrogen respiration and protein metabolism are increased simultaneously.

The course of nitrogen metabolism in fully manured and nitrogen-deficient leaves is normal as Richards and Templeman (1936) have shown, the relative concentrations of the nitrogen fractions remaining unchanged during the leaf succession.

In the potassium-starved leaf the nitrogen metabolism undergoes a manifest change. Here protein is at the time of emergence of the leaves at least as high as in the normal leaf, the mean protein level in all leaves being the same as that of fully manured plants (Table XVI). At the same time a high amino-N content is found, and, as Richards and Templeman showed, a high amide content also. In these leaves sugar concentration is always low, primarily due to low assimilation rate (Gregory and Baptiste, 1936). The evidence from development of plants deprived of potassium suggests that protein synthesis goes on very rapidly: a supernormal tiller production is found, leaf production rate is normal, and the leaves are of normal size. Meristematic activity in such plants is very high. These statements apply to the high sodium conditions used in these experiments: with high calcium and low phosphorus supply conditions are somewhat different, and a preliminary statement has been published on this point by one of the present authors (Gregory, 1937). Richards and Templeman have suggested that potassium supply has no direct effect on protein synthesis. On the contrary the low level of sugar may in large part be due to the rapid utilization of sugar of photosynthesis in protein production, and no accumulation of sugar results. It may even be that the first use for sugar is in protein synthesis (Gregory and Baptiste, 1936).

The proteins of the potassium-starved leaf are very unstable, very rapid death of individual leaves occurs, and a rapid fall of protein level during senescence (Richards and Templeman, 1936). If amino N results from protein degradation rapid production of amino acid by protein break-down would be anticipated. If protein were resynthesized from amino acid the accumulation of the proteolytic products would tend to reverse the action and levels of protein and amino N would vary in proportion. This might be countered by rapid translocation away of amino N, but this on the other hand would lead to a relatively low ratio of concentration of amino N to protein. In fact the reverse appears, and accumulation of amino N is seen. This would suggest that the route of protein synthesis is distinct from that of protein degradation, and that the rates of these processes may vary independently, and in fact that a cyclic process is operating. Variation in velocity of the reactions concerned would lead to accumulation: thus if the synthetic route to protein is rapid protein tends to accumulate, and by mass action increases the break-down to amino acid. If the deamination process is the slowest then amino N will accumulate. The very high respiration rate of the potassium-starved leaf is undoubtedly associated with this accumulation of amino N, but, as indicated above, the part played by amino acid in stimulating respiration is not yet clear. It may reasonably be assumed that deamination occurs with liberation of ammonia, and the finding of Richards and Templeman (1936) that the amide accumulates in the potassium-starved leaf proportionally with amino acid is in no way unfavourable to this view. Apparently the ammonia is metabolized to amide by utilization of carbohydrate, but details of this process are not clear.

In these experiments as potassium starvation increases respiration passes through a maximum, and this is true of all leaves in the succession after the third, as Richards (1932) first showed and is here confirmed. This has already been described as due to the level of carbohydrate. Feeding experiments performed in this Institute with potassium-starved leaves (Said, 1934) have shown that potassium supplied alone has no effect on respiration of such leaves, but if given with sugar immediate increase in respiration results. The leaves in these experiments were isolated so that amino acid was not translocated away, but since high respiration followed sugar feeding this suggests that oxidative deamination is not alone concerned.

The theory of preferential oxidation of carbohydrate is closely concerned in this connexion. The following considerations must be borne in mind. If carbohydrate is preferentially respired, while sugar supply in potassium deficiency is low through failure of photosynthesis, how, on this assumption, is protein synthesis maintained at a level at least as high as in the fully manured leaf, in spite of low carbohydrate concentration? Again regarding the suggestion of James (McKee, 1937) that the inhibition of protein oxidation by carbohydrate results from competition for carboxylase between products of deamination and glycolysis, this might account for the rapid oxidation of proteolytic products in the potassium-starved leaf but not for the subnormal respiration rate of the nitrogen-starved leaf, where carbohydrate concentration is high and amino acid very low. Clearly other factors are involved beside level of carbohydrate. The bearing of oxygen tension on the activation of the proteolytic enzymes which Mothes (1933) investigated does not appear here to be very close. Protein degradation in the potassium-starved leaf goes on in full light, and in this respect the greater importance of carbohydrate, as Paech (1935) insists, seems justified.

It would appear that the conditions in the potassium-deficient leaf are somewhat as follows. Rapid synthesis of protein occurs and at the same time rapid protein degradation, the two sets of processes being distinct. By deamination ammonia is set free and amide synthesized from carbohydrate through organic acid formation. The carbon residues of deamination may perhaps be completely respired away. From amide by a different route through amino acid, or directly from amide, protein is resynthesized. In the K-deficient leaf protein level is normal and amino N much increased, the protein cycle must therefore be traversed more rapidly than normally. The output of CO_2 is thus related to the more rapid rate at which the cycle is traversed. This same hypothesis would equally well account for the situation in the nitrogen-starved leaf. Here the cycle is very slow, and the output of CO_2 low in spite of high concentration of sugar. In the fully manured plants during the progress through the successive leaves, protein metabolism declines, and in step with this the respiration falls in spite of accumulation of sugar in the later leaves. Tentatively, therefore, the suggestion is put forward that the carbohydrate oxidation in the green leaf is regulated by the rate of protein synthesis. The production of CO_2 may or may not be due mainly, in the living leaf, to the process of deamination, but even if carbohydrate respiration is the main source of CO_2 output, the intermediate products being utilized through organic acid formation in protein synthesis, yet there is a general 'regulation' at work which controls the whole process, though the nature of this is at present quite obscure. Regarded from the standpoint of efficiency, this is highest in the fully manured leaves, for here the loss of carbon as CO_2

per unit of protein present is least; all deficiencies lead to a greater loss of CO_2 on this basis, as was shown above.

Regarding protein as the hypothetical source of all carbon lost in the plant, it may be of interest to calculate the amount of protein breakdown necessary to account for the CO_2 output. Using the figures in Table XVI, and selecting the series with highest mean respiration rate (N_1K_3); the mean protein-N content is 3·32 per cent. of the dry weight, equivalent to approximately 20 per cent. protein, or 100 mg. carbon capable of producing 370 mg. CO_2 per gm. dry weight. As the mean respiration rate is 7·86 mg. per gm. dry wt. per hr. this corresponds to a loss of 2·1 per cent. of the protein content per hour. Even without taking into account protein synthesis which may be occurring concurrently, this value is not incommensurate with those actually found during proteolysis of the darkened leaf.

The foregoing discussion is intended primarily to elucidate the experimental data given in this paper, and seeks to emphasize the close connexion between carbohydrate and nitrogen metabolism.

In conclusion, the authors wish to thank Sir E. J. Russell for the facilities to carry out this investigation at Rothamsted, to Dr. R. N. Mathur for the estimations of the nitrogen fractions carried out under the direction of Mr. F. J. Richards, to whom also we owe much in discussion, and to Prof. V. H. Blackman for unfailing interest.

SUMMARY

1. Barley (var. Plumage Archer) was grown in sand culture. Two main manurial series were used: (1) with maximal nitrogen and increasing deficiency of K—N_1K_1 control, N_1K_3 with $\frac{1}{9}$ max. K, N_1K_5 with $\frac{1}{81}$ max. K. (2) Maximal potassium and increasing deficiency of N—N_3K_1 with $\frac{1}{9}$ max. N, N_5K_1 with $\frac{1}{81}$ max. N. In addition, one series N_5K_5 in which both N and K were at $\frac{1}{81}$ of the maximum level. Each treatment used was replicated 80–120 times and in all 600 pot cultures were employed.

2. Respiration was estimated by absorption of CO_2, the apparatus which embodies new features is fully described.

3. As each leaf on the main axis reached maturity its respiration rate was determined; a later stage was also investigated. Sufficient leaves of a particular status were collected at random from each manurial series on each occasion to give 3 gm. fresh material. In all 132 determinations were carried out.

4. On parallel samples of leaves determinations of reducing sugar and sucrose were made on alcohol extracts of fresh material by the Hagedorn and Jensen method. Estimations of the following nitrogen fractions were made on a third sample: total nitrogen, crystalloid N, protein N, total amino N. The methods of Richards and Templeman (1936) were employed.

5. Water content of leaves again showed a reduction in N-deficient and a large increase in K-deficient leaves. Cf. Gregory and Richards (1929), Richards (1932).

6. Respiration rate is greatly reduced in the N-deficient, and greatly increased in the K-deficient series. The mean values for all successive leaves at emergence is given below in mg. CO_2 per gm. dry wt. per hr. are as follows:

N_1K_1 6·61	N_3K_1 4·92
N_1K_3 7·71	N_5K_1 4·65
N_1K_5 7·37	N_5K_5 4·60

Respiration rate increases with K deficiency to the K_3 level and then falls on further reducing K supply. The optimal value at the K_3 level is shown by each leaf in turn, both at first emergence and at later stages. Richards' (1932) findings are thus confirmed.

7. Respiration rate in the successive leaves reaches a maximum in the early leaves, falls to a minimum, and then increases in the last leaves. The details which differ in the manurial series are fully described.

8. Respiration drift in the dark varies with manuring and with the leaf succession. In the control series the early leaves show a rapid decline in respiration: this effect reaches a maximum in the fifth leaf. In the K-deficient series the maximum effect occurs in the fifth leaf (N_1K_3) and sixth (N_1K_5), and in later leaves disappears, no fall in respiration occurring in six hours. In the N-deficient series only the third leaf in the N_3K_1 series shows such a fall, while later leaves in this series, and all leaves in the N_5 series, show no fall in respiration. In the N_5 series respiration remains constant for sixty-eight hours.

9. For all leaves the mean values of the sugar fractions (given as hexose as per cent. dry wt.) are as follows:

Reducing sugar.		Total sugar.	
N_1K_1 2·19	N_3K_1 1·21	N_1K_1 15·6	N_3K_1 21·8
N_1K_3 1·58	N_5K_1 1·33	N_1K_3 12·7	N_5K_1 18·9
N_1K_5 1·37	N_5K_5 1·29	N_1K_5 5·5	N_5K_5 19·1

10. The corresponding nitrogen fractions as per cent. dry weight are:

Protein N		Amino N.	
N_1K_1 3·27	N_3K_1 1·70	N_1K_1 0·273	N_3K_1 0·148
N_1K_3 3·32	N_5K_1 1·24	N_1K_3 0·379	N_5K_1 0·104
N_1K_5 3·32	N_5K_5 1·21	N_1K_5 0·464	N_5K_5 0·091

Potassium deficiency leads to much reduced sugar, to normal protein, and to much increased amino acid. Nitrogen deficiency leads to much increased sugar, much reduced protein N and amino N.

11. High respiration rate is associated with low sugar and high amino N in the K-deficient series: low respiration accompanies high sugar and low nitrogen fractions in the N-deficient series.

12. The results are examined statistically and the correlation coefficients, multiple regression coefficients, and regression equations are given. In the K_5 series with lowest potassium, sucrose content is the determining factor in respiration, in the K_3 series sucrose and protein N are significantly correlated

with respiration; in the N-deficient series sugar content is uncorrelated, but protein N and amino N are highly correlated with respiration. In the control series amino N shows the highest correlation with respiration.

13. The results are discussed in relation to the source of CO_2 in aerobic respiration. A schema is presented indicating the close relation of respiration and nitrogen metabolism. It is suggested that the CO_2 production is related to the rate at which a cycle of protein synthesis and proteolysis proceeds, the intermediate products of glycolysis being drawn into this metabolic cycle, and glycolysis is 'regulated' by the utilization of carbohydrate in this way.

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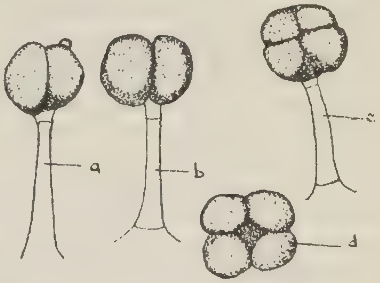
NOTES

BROM-THYMOL BLUE IN AQUEOUS SODIUM HYDROXIDE AS A CLEARING AND STAINING AGENT FOR FUNGUS-INFECTED ROOTS.—Recent work with various root-infecting fungi has called for some method of rapidly examining seedling roots for fungus infection. A method of clearing and staining whole roots for mounting directly under the microscope is often needed, not only for inspection of the roots of young plants from the field, but also for the recording of infection in the roots of experimental plants. A solution of 0·04 per cent. brom-thymol blue in 4 per cent. (or 'Normal') aqueous sodium hydroxide has been found to fulfil these requirements. The roots are allowed to soak in this solution for a period of not less than five minutes, and are then mounted in the same reagent. In addition to its clearing effect, the sodium hydroxide has a macerating action, which enables the root to be pressed out flat under the coverslip, thus facilitating microscopical examination. Young fungus hyphae, spores, and sporangia are stained blue in virtue of their high protoplasmic content, and are hence easily seen through the cleared root cortex. The meristematic tissue of the root apices also takes up the blue stain. Old fungus hyphae are not stained, but are seen more easily in such preparations owing to the clearing and macerating action of the reagent. Material pickled in 70 per cent. alcohol appears to respond as well to the treatment as does fresh material; preparations made in this way cannot be kept for permanent record owing to the continued action of the reagent on the tissues.

S. D. GARRETT.

ROTHAMSTED EXPERIMENTAL STATION
HARPENDEN, HERTS.

PIGMENT GLANDS OF THE TOMATO.—In the 'Annals of Botany' a note was published in 1933 (vol. xlvii, p. 913) indicating that the pigment citrinin first described by Hetherington and Raistrick as a metabolic product of *Penicillium citrinum* also occurred in the flowering plant *Crotalaria crispata*. Citrinin is a pale yellow acid, insoluble in cold water, dissolving in dilute sodium carbonate with evolution of carbon dioxide to form a deep orange-yellow solution, brownish red when concentrated. The pigment is reprecipitated on the addition of dilute acid. The change of coloration is very pronounced. The pigment is soluble in chloroform, acetone, and hot alcohol, and the alkaline solution when warmed with iodine gives the iodoform reaction.



Surface glands of the Tomato.—(*a*) Gland showing liquid exudation from one of its four cells. (*b*) Gland seen in side view. (*c*) Gland viewed obliquely. (*d*) Gland viewed from above.

From time to time, various native plants have been investigated for the presence

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of this pigment without success. In *Penicillium citrinum* the optimum temperature for its production is rather high, and its occurrence in a tropical plant corresponds with this fact. Recently, some evidence has been obtained of its presence in a plant with a high temperature optimum, namely, the tomato.

Any one who has handled tomato plants knows that the surface is sticky, and after long handling of clean plants the fingers become covered with an appreciable, pale yellow, sticky film. This dissolves in dilute sodium carbonate, forming a deep yellow solution. The surface of the leaf and stem is covered with numerous shortly stalked, quadricellular, sticky glands, in addition to longer ordinary hairs (see sketch on p. 563). The glandular hairs turn brown in dilute sodium carbonate and the colour slowly diffuses out.

A quantity of fresh tomato leaves was dried rapidly, powdered, and sieved, the finer dust being used for extraction. Three methods of extraction were used:

1. The dust was directly extracted with dilute sodium carbonate. On precipitating the filtrate with acid, the dried brown residue was extracted with alcohol. On evaporating to dryness, a very small amount of yellowish residue was left which gave the iodoform test.

2. The dust was first extracted with water, the water squeezed out, and the solid residue extracted with sodium carbonate, precipitated with acid, and the dried brownish acetate extracted with acetone. The extract was very small in amount, pale yellow, and gave the iodoform test.

3. The dust was directly extracted with acetone, the filtrate evaporated to dryness, extracted with sodium carbonate, and the filtrate precipitated with dilute hydrochloric acid and again filtered. The pale yellow residue dissolved in sodium carbonate to form a deep orange-yellow solution which gave the iodoform reaction.

A convenient method of obtaining the pigment directly from the glands is by drawing fresh stems and leaves of the tomato through the hands until they are quite sticky. On washing the hands in dilute sodium carbonate and repeating the process several times, the liquid becomes deep brown in colour. On adding acetic acid, if the liquid has been kept as far as possible out of contact with the air during extraction, a bulky, pale precipitate is formed which gives reactions characteristic of citrinin. In alkaline solution, however, the pigment obtained from the tomato appeared to undergo rapid oxidation to an insoluble dark brown compound. Hence, on attempting to purify by dissolving in alkali and reprecipitating by acid, with each extraction, the liquid became paler until all the pigment had been converted into the insoluble brown oxidation products. That the glands do actually contain an oxidase enzyme was shown by the fact that scrapings from the surface of the leaves and stem after soaking in warm water gave a distinct reaction with tetramethylparaphenylendiamin (ursol) tartrate.

Apparently the glands of the tomato contain a pigment similar to or identical with citrinin. As extracted from the tomato it has, however, the peculiarity that in alkaline solution it undergoes rapid oxidation to an insoluble brown compound. No definite evidence of its presence could be obtained from parts of the plant other than the glands.

A. J. EWART.

UNIVERSITY OF MELBOURNE.

THE EFFECT OF AGE ON NET ASSIMILATION AND RELATIVE GROWTH RATES IN THE COTTON PLANT.—The changes which occur with age in the physiological processes of the plant constitute a subject of considerable interest. Information as to some of these changes may be obtained by the study of derived data,[1] calculated from such primary data as weights of the several parts of sample plants taken at intervals through the growing season.

The results here recorded apply to the vegetative period before first flowering. They were obtained from random plant samples taken at fortnightly intervals from cultivation experiments carried out in seasons 1933–4 and 1934–5 at Barberton, South Africa. These experiments are described in detail elsewhere.[2] Since no differential effect of age as between treatments is found for net assimilation or relative growth rates, the means of all the three cultivation treatments employed will be considered here.

For each season the following statistically significant effects are found: Net assimilation rates, calculated by Gregory's method, but on a basis of leaf weight instead of leaf area, show no general rise or fall up to time of flowering. This constancy of rate confirms the findings of Gregory for barley, in which also during the vegetative phase net assimilation remains constant. On the other hand, both relative rate of total dry weight increase (efficiency index) and relative leaf growth-rate have a slight downward trend in time, as is shown by the negative quadratic regressions for the Naperian logarithms of total dry weight and leaf dry weight respectively.[3] The percentage of the total dry weight which consists of leaves also falls with time, as is indicated below:

Leaves as a Percentage of Total Dry Weight of Shoot

Season.	Initial sample.	At flowering.
1933–4	77·0	54·7
1934–5	74·9	56·4

Since both efficiency index and relative leaf growth-rate fall with time as stated above, it is of interest to compare their average rates of fall, i.e. the quadratic regression coefficients. This comparison is shown below:

Mean Quadratic Regression Coefficients (14-day) for Logarithms of Total Dry Weight and Leaf Dry Weight

Season.	Total weight.	Leaf weight.	P*
1933–4	−0·0587	−0·0751	<0·01
1934–5	−0·0464	−0·0545	0·1−0·2

* P = Probability of the difference between the two measures being due to chance.

In 1933–4 the relative leaf growth-rate fell more rapidly than the efficiency index. The similar effect in 1934–5, although failing to reach the conventional level of significance, is very probably real, confirming as it does the 1933–4 result.

These results may be explained as follows: Net assimilation rates per unit leaf weight up to first flowering have been found to have no significant general trend in time. Relative leaf growth-rates, on the other hand, fall significantly with time. If net assimilation rate has no downward trend, though this is not proved, the fall in relative leaf growth-rate implies that an increasing proportion of the products of photosynthesis as time proceeds is used for stem, flower-buds, &c.; the ratio of leaf weight to total dry weight falls with time as a consequence. The efficiency index also falls with time, since a decreasing proportion of the plant weight consists of assimilating material. The rate of fall of efficiency index, however, is found to be less than that of relative leaf growth-rate, because the increasing proportion of assimilates going to the stem, &c., is included in the total dry weight and not in the leaf dry weight.

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[1] F. G. Gregory, Ann. Bot. xl. 1, 1926.

[2] O. V. S. Heath, Journ. Agric. Sci. In Press.

[3] O. V. S. Heath (loc. cit.).

It is of interest to note that an explanation similar to the above may be applied to Crowther's results[1] for the effect of nitrogen on relative leaf growth-rate, &c. He finds that: 'nitrogen has a predominant effect on relative leaf growth-rate and a much smaller effect on efficiency index. Net assimilation rate is unaffected by either water or nitrogen supply from the earliest samples up to the time of maximum leaf weight.' Since nitrogen increases the relative leaf growth-rate but has no effect on net assimilation rate (per unit leaf weight), a smaller proportion of the assimilates must go to stem, &c., and therefore the leaf weight percentage of total weight must increase.[2] This will cause an increase in the efficiency index, but not so great as that in relative leaf growth-rate, since the extra proportion of assimilates which goes to stem, &c. (i.e. to total but not to leaf) without nitrogen will go to leaf with nitrogen (i.e. to total *and* to leaf).

O. V. S. HEATH.

Research Institute of Plant Physiology,
Imperial College of Science and Technology, London.

A SIMPLE DEVICE FOR THE AUTOMATIC CONTROL OF A STREAM OF AIR PRODUCED BY AN ORDINARY SUCTION PUMP.

—A supply of air is a common need in physiological work and may be met, readily and cheaply, by the employment of the water suction pump. Its use for purposes where constancy of air flow is desired has, however, serious drawbacks. The water flowing through the pump is subject to variations produced by alterations of pressure in the water mains and by the manipulation of any tap in the vicinity. This not only causes great fluctuations in the rate of air flow but frequently induces the calamity known as 'sucking back'. The arrangement to be described here enables a stream of air to be produced on which variations in the water flow have comparatively little effect.

The method is largely dependent on the efficient working of the automatic control valve shown in the diagram. This consists of a U-tube into one arm of which is inserted, by means of a rubber bung, a piece of glass tubing (*d*) regularly drawn out at *s* to a tube (*g*) with a much narrower bore. Inside this is situated a valve (*v*) made from a length of glass tubing (or rod) with a diameter no more than half that of the valve guide (*g*). It is expanded at its upper end and finely ground with emery powder and water to make a good seating with *s*. For best results this seating should be uniform and not more than a twelfth of an inch wide. The valve is worked by a light glass float (*f*) of a good but easy fit resting on mercury in the U-tube. To the same arm of the U-tube is fitted a side tube (*s.t.*) which leads to a three-way piece (*t*). The other two arms of *t* communicate respectively with the pump (via a water trap) and with the system through which it is desired to pass the constant air-stream (*c*).

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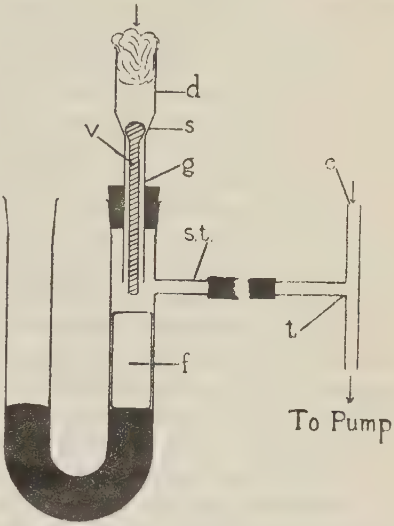
[1] F. Crowther, Ann. Bot. xlviii. 877, 1934.

[2] Measurements based on Fig. 2 of Crowther's paper confirm this.

The principle of the apparatus is as follows: When the pump is working it is arranged that the reduced pressure is such as to cause the mercury to rise sufficiently to press the float on to *v* and open the valve. Air is now being sucked in past the valve and also at *c*. If the suction is now increased the mercury will rise further in the arm of the U-tube and the valve opening will be enlarged, its resistance to the passage of air will consequently decrease, and not only will more air enter but a greater proportion of the total quantity entering the system will come by this route. On the other hand, if the suction diminishes the mercury will fall and the valve tend to close, thus increasing its resistance to the passage of air so that there will now be a greater proportion of the total reduced suction exerted at *c*. By this means fluctuations in the pump do not affect the stream of air passing through *c*, which remains relatively constant, but are dealt with by automatic regulation of the valve opening.



It was found that for best regulation there must be a comparatively high resistance in the constant flow lay-out. This was provided by the incorporation of a capillary stop-cock (henceforth referred to as *r*) through which the air was made to flow before passing through a water flow-meter, and by means of which a varying degree of resistance could be obtained at will by opening or closing the tap. If a good tap grease be used this resistance is capable of careful regulation and is much more static than the usual rubber tubing and screw-clip. It is frequently convenient to employ *r* as a fine adjustment enabling the air-flow to be brought to any desired rate, but it must be borne in mind that its function is primarily to provide a resistance and as such it must never be completely open. If it is found that a very much greater rate of flow is required this can readily be brought about by raising the tube *g* or by removing mercury from the U-tube.

For short periods of a few hours it is possible to produce a stream of air in which no fluctuations can be detected by a water flow-meter. Even with longer periods the constancy leaves little to be desired as the following examples will show: In one case a flow of 25·2 litres per hour was maintained for five days to within ·2 per cent. without any adjustments whatever. In another instance no measurable alterations in a flow of 1·5 litres per hour occurred over a period of three days. This is in striking contrast to the state of affairs without such automatic regulation. These two examples by no means represent the limits of flow that can be so controlled. That will depend, amongst other things, on the height the mercury travels before the valve opens and the efficiency of the suction pump.

In addition to the importance of having a relatively high resistance in the constant air-stream, there are one or two other points to which attention must be paid if the apparatus is to work satisfactorily. The efficiency of the valve should be checked by varying the suction produced by the pump. With the best valves it was found that the water flow could be varied from a position in which it would normally produce a negative pressure of 30 cm. of mercury to full on (74 cm. mercury) with but a momentary variation in the flow-meter reading. For such results the valve must be light and easily worked by the thrust of the mercury on the float. Should it show any tendency to stick it may be overcome by covering with cycle-valve rubber, but a direct ground glass seating is more satisfactory, and for best results another valve should be made with less seating area. Such 'sticking' is not infrequently caused by the presence of particles of dust drawn in with the air current, and this should be obviated by inserting a plug of cotton-wool in the tube *d* above the valve.

Dust and moisture must also be prevented from accumulating in the resistance tap *r* and the capillary of the flow-meter, otherwise their resistance to the passage of air will increase and the rate of flow thus fall. The practice adopted was to pass the air through strong sulphuric acid and a U-tube of calcium chloride plugged with cotton-wool before reaching *r*.

The above arrangement has proved itself to be of great efficiency in cases where continuous aeration was required. The number of units to be aerated necessarily imposes a limit on the constancy to which the flow can be maintained over long periods without minor adjustments. Nevertheless, a series of eighteen flasks has been successfully aerated over a period of three days with only comparatively minor fluctuations and without showing any tendency towards 'sucking back'. Such a performance was found to be impossible using the pump directly, even if great constancy of air-flow was not required.

Other methods of control may be in use in other laboratories, but it is thought that such an efficient and simply constructed device is worthy of record.

G. F. ASPREY.

BOTANY DEPARTMENT,
GLASGOW UNIVERSITY.

Studies in Vernalisation of Cereals

I. A Comparative Study of Vernalisation of Winter Rye by Low Temperature and by Short Days

BY

O. N. PURVIS

AND

F. G. GREGORY

(*From the Research Institute of Plant Physiology, Imperial College of Science and Technology, London*)

With Plate XXII and six Figures in the Text

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INTRODUCTION

AS applied to the temperate cereal plants, vernalisation is a well-known technique by which flower production and fruiting is accelerated by application to the germinating grain of temperatures slightly above freezing-point. The practical application of the technique, first introduced by Russian agronomists, is fully described in the bulletins of the Bureau of Genetics (1933 and 1935) in which also much of the earlier experimental work directed towards the elucidation of the nature of the process is discussed. One of us also has elsewhere (Purvis, 1936) given a short account of the practical and theoretical aspects of this problem.

In a previous paper in this journal an account was given of an analysis of

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the effect of low temperature during germination on the subsequent development of the cereal plant, more especially of winter rye; it was there shown that length of day is interrelated with conditions of germination in determining the subsequent flowering behaviour. From the outset of that investigation the importance of length of day was well realized, whereas this interrelation had not in the past been given sufficient consideration by some investigators. This omission is reflected in certain inadequacies in the hypothesis which Lysenko has put forward to explain the mechanism of vernalisation, an hypothesis which will be dealt with later in this paper. The importance of the photoperiodic relations had, however, already been noted (Maximov and Pojarkova, 1924; Pojarkova, 1927; Lebedinceva, 1933; McKinney and Sando, 1933 and 1935) and McKinney and Sando had also in other respects reached similar conclusions. In the previous paper referred to (Purvis, 1934) no mention was made of their work as, unfortunately, it was then unknown to the writer. The interaction between vernalisation and length of day has also been studied by Harder and von Denffer (1937).

In spite of the very considerable body of experimental work directed to the elucidation of the physiological processes involved in vernalisation, the proximal cause of this effect of low temperature during germination is by no means clear. In this and subsequent papers experiments are described which have been carried out in this Institute with a view to a closer analysis of the phenomena.

HISTORICAL

The phenomenon of vernalisation marks the focal point of two quite separate lines of investigation, one deriving from the work of Gassner on the influence of temperature during germination in cereals, the other from the photo-periodic studies of Garner and Allard.

Gassner had clearly demonstrated the relationship of sowing date and of temperature during germination to the subsequent flowering of winter cereals, and endeavoured to explain his results in terms of a specific 'cold requirement' of such winter varieties to which spring varieties are not subject (Gassner, 1918). This was inadequate to explain all the facts and a knowledge of the subsequent work on photo-periodism (Garner and Allard, 1920 and 1923) led to the demonstration by Purvis (1934) that under short-day conditions winter rye and other cereals remained in an apparent vegetative state, even though germinated at 1° C. Further, by direct dissection of the growing-point it was revealed that the continued *vegetative* condition of winter rye under short-day conditions, whether germinated at 1° C. or at 18° C., is apparent only, for flower differentiation occurs in due course; it is elongation of the stem and with it ear emergence that is suppressed.

A further fact established very clearly was the relation of leaf number to the occurrence of flowering. Spring rye normally produces seven or eight leaves on the main axis before lateral initials give rise to groups of flowering spikelets, and this number is independent of the temperature during germination; in winter rye the leaf number is determined by the germination temperature. In both varieties, however, in short days the leaf number exceeds twenty before flower differentiation begins. McKinney and Sando also noted the variation in leaf number in relation to the length of day.

It is then clear that low temperature of germination cannot be regarded as the initiating factor in flower production but is rather an accelerator. The work of Rasumov has shown that exposure to short days in the early stages of growth in millet accelerated flowering under subsequent long days, although the plant is a typical short-day plant in its flowering behaviour (Rasumov, 1930). This phenomenon he called photo-periodic induction. A somewhat similar effect of short days in accelerating flowering has been found in winter rye, and will be fully discussed later. Growth rate of the meristem is actually more rapid in short than in long days (Purvis, 1934), but, as stated above, continued short-day treatment prevents emergence of the ear, with the result that flowering can eventuate only in long days. If, however, an initial period in short days is followed by long-day treatment the more rapid differentiation results in earlier flowering than occurs in long days alone. This effect of short days was independently observed by Lebedinceva (1933), McKinney and Sando (1935), and by the present authors (1934 and 1936). It has been shown (Oleinikova, 1933) that the flowering may be accelerated in 'unvernalised' cereals by a succession of day lengths from short to long (as in spring), but a succession from long to short (as in autumn) was without effect. Thus may be explained the rapid flowering of early sown winter cereals which have been protected from chilling.

In view of these facts the theory of Lysenko (Bull. 17, p. 9) may be briefly examined. This involved the concept of a series of stages in the progress toward flower development, each one depending for its inception on the completion of the preceding stage. In the case of temperate cereals the first of these stages depends on low temperature, and Lysenko considers that unless this stage is completed no progress at all can be made towards flowering. If this is so, under no circumstances whatsoever should winter rye flower unless the seed is subjected to low temperature for a certain definite period. There is ample evidence that this is not the case. In the first place, it has already been shown that preliminary treatment by short days without low temperature accelerates the flowering of winter rye. Again, in the absence of any accelerating factor, winter varieties do not make indefinite vegetative growth but come to flower under long days. It has been shown (Lojkin, 1936) that the wheat varieties 'Leaps Prolific' and 'Turkey Red', when sown untreated, come to ear in approximately 150 long days, and flowering has been observed in five wheat varieties grown under continuous light at 20° C. in little over 150 days (Vasiliev, 1934). In field experiments on vernalisation planting is usually late (May) to avoid chilling by spring frosts, and, as a result of the long vegetative period, the plants are not ready to flower until

October, when the natural day-length inhibits the final emergence of the ear. Thus, flowering is considered to have failed, but an examination of the growing-points at this date reveals that in many cases flower differentiation has already taken place (Purvis, 1934). In our experiments Petkus winter rye, sown unvernalised on March 17, 1936, under a day-length increasing from twelve hours, came into ear in about 170 days and produced fertile ears and long straw, showing that failure to ear in plants sown in May is due to shortening days at the time at which the 'shooting' stage is reached. Furthermore, it will be shown in this paper that the effect of low temperature during germination is proportional to the duration of the treatment up to an optimum degree of acceleration.

In winter cereals flower initiation does not require the action of any special external factor, but it can be accelerated by either low-temperature germination or by an initial period of short days. In fact, if vernalisation is defined as a treatment in early stages of growth which shortens the vegetative phase one may speak legitimately of vernalisation by short days as well as of vernalisation by low temperature. It must, however, be emphasized that in either case a period in long days is necessary for flowering to be completed, and for this reason the winter cereals may retain their status as 'long-day' plants.

THE RELATIONSHIP BETWEEN THE DURATION OF LOW-TEMPERATURE TREATMENT AND RATE OF PROGRESS TOWARDS MATURITY

Experimental procedure.

In 1935 seeds of Petkus winter rye were germinated in moist sand at a temperature between 1° and 2° C. for periods ranging from four to forty-nine days. The sowing dates were so arranged that all these periods terminated on May 14 (with the exception of the forty-nine days' period which ended one week later), and on that date the seedlings were planted out in sand cultures.[1] A record for each plant was made of the number of leaves produced on the main axis prior to flower production and of the date of anthesis.

In 1936 a further experiment was carried out to confirm and amplify the information gained in the previous year. The seed was germinated in moist sand for periods ranging from five to nine weeks and also for a short period of four days, sowing being timed to allow all the seeds to be planted out

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[1] The plants were grown in unglazed earthenware (12″) pots containing washed sand. Nutrients were added in dilute solution through a small pot embedded in the sand, and each pot received the following total weights of salts given in eight weekly doses.

Disodium hydrogen phosphate	2·5 gm.
Calcium chloride	0·4 ,,
Magnesium sulphate	1·3 ,,
Sodium nitrate	9·1 ,,
Potassium sulphate	2·1 ,,

In addition each pot received weekly 1 c.c. of a saturated solution of ferric chloride containing 1 per cent. manganese sulphate, given twenty-four hours after the other nutrients.

together. In addition some of the seedlings which had already received nine weeks' chilling were planted out after further periods of five to eleven weeks at the low temperature. These later-planted variants are not strictly comparable with the others as the later stages in their development took place under a decreasing day-length, but if this fact is kept in view they yield useful information. In 1936 the sand-culture method was replaced by soil cultivation in 12″ pots, but the changed method yielded no advantage either in quality of plants or reduction of labour.

Experimental results.

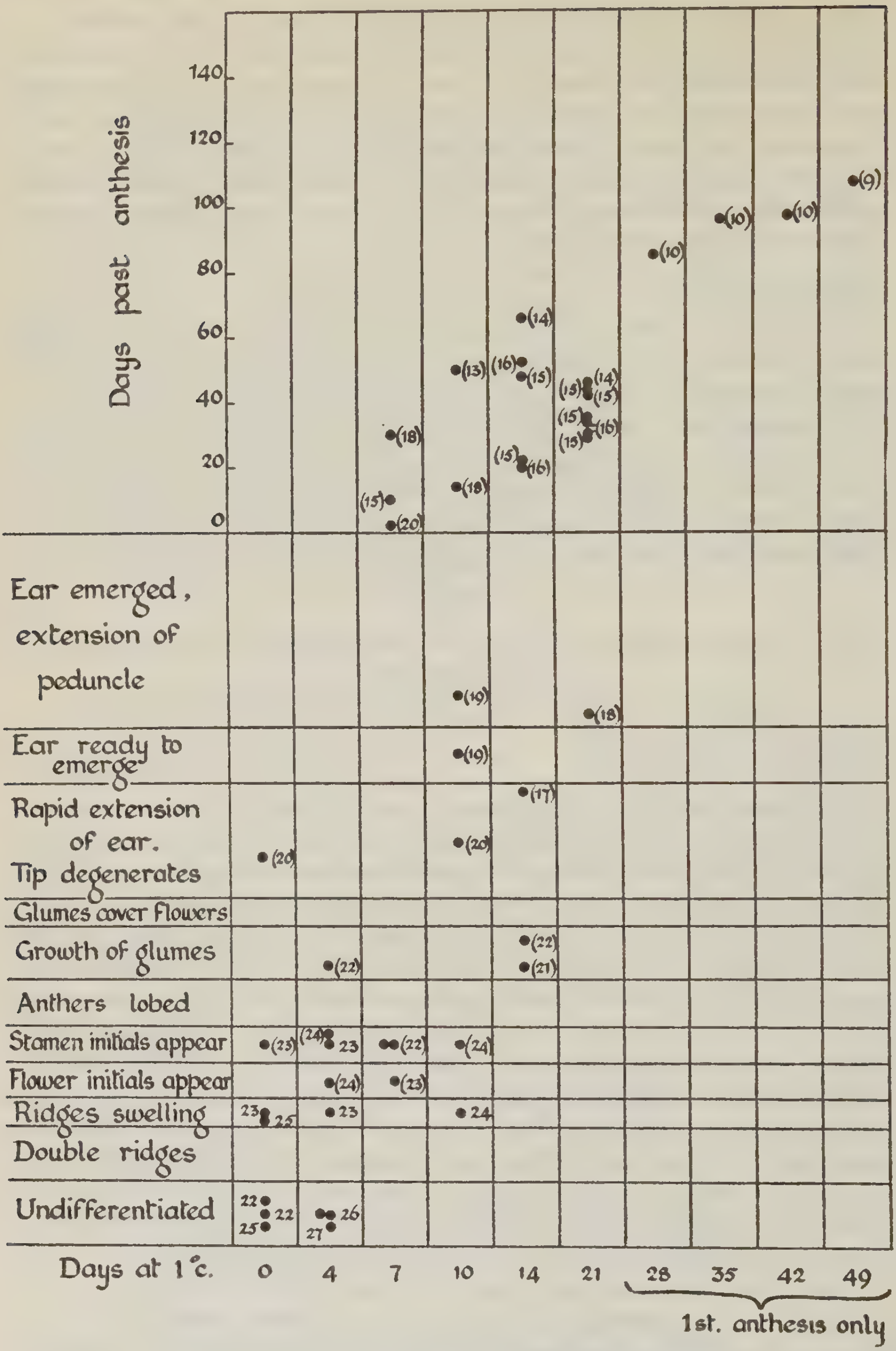
The time from planting to flowering in both experiments varies with the duration of exposure to cold, showing that incomplete vernalisation can occur. Partial vernalisation, however, has the effect of accentuating the variation in individual response of the plants, and this effect renders difficult the presentation of the results obtained. With some treatments one or two plants did not reach the stage of anthesis before the experiment was ended in October, and in consequence no definite average time to anthesis was obtainable. In Table I the time elapsing between planting and the first anthesis is given for each series of the 1935 experiment, and also the total time to anthesis from the beginning of vernalising treatment. It is clear that quite short exposures to cold, such as seven or eleven days, had some effect in curtailing the vegetative period and that this effect increased progressively with the duration of treatment. It is to be noted that the *total time* from the breaking of seed dormancy to anthesis is nearly constant for treatments of eleven to forty-nine days' duration. The significance of this fact will be considered later.

TABLE I

Relation between Duration of Treatment and Length of Vegetative Period. 1935 Experiment

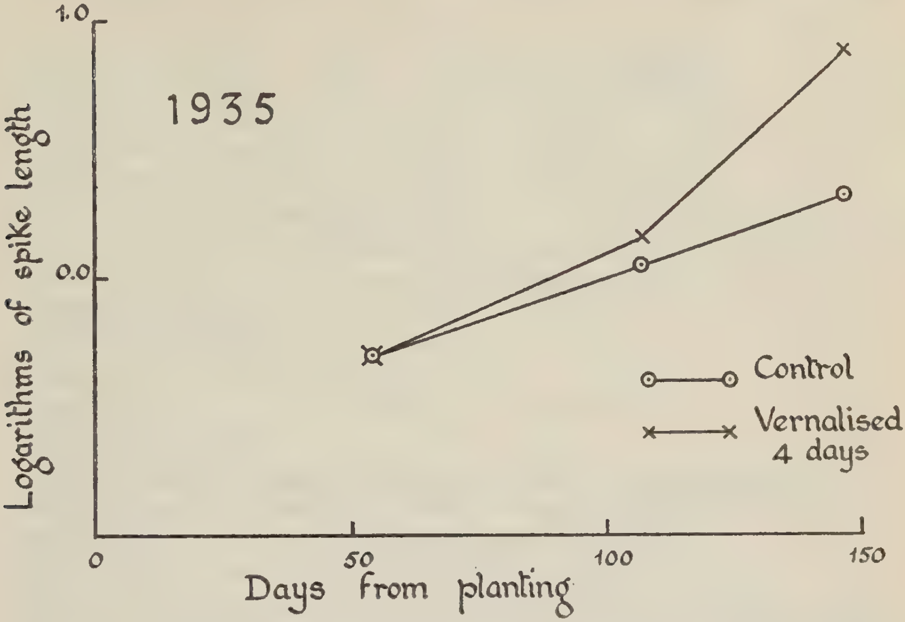
Days of treatment	0	4	7	11	14	21	28	35	42	49
Days from planting to anthesis	—	—	130	91	87	76	67	55	56	49
Days from sowing to anthesis	—	—	137	102	101	97	95	90	98	98

A more precise representation of the effect of these treatments is given in Text-fig. 1, which has been constructed in a manner similar to that of the diagrams used by workers at Wageningen (Blaauw, 1924). In early October the plants which had not flowered were dissected and the growing-points of the main axis examined. In Text-fig. 1 the abscissae represent the duration of the low-temperature treatment, while the ordinates represent the relative duration of successive stages of spike differentiation assessed from the results of a number of previous experiments. An exception to this is the first stage, preceding any morphological sign of spike differentiation, which actually covers a somewhat longer period than all the subsequent stages taken together. For convenience this stage is curtailed in the graph. Each plant examined is



TEXT-FIG. 1. Diagram of winter rye vernalised for different periods, showing condition of growing-point 22 weeks after planting. Each plant examined is represented by a dot, and the numerals represent leaf number at time of examination: when the full complement of leaves is then present the number is enclosed in brackets.

represented in the figure by a point placed in the rectangle corresponding with the stage in development it had attained and the treatment it had received. When anthesis had already occurred the number of days since that event is recorded by the position of the point. The general trend of the points gives a clear picture of the relationship between treatment and progress towards flowering; a progressive increase in the extent of 'vernalisation' from seven days' treatment upwards is shown, but four days' chilling appears to have no effect. The numbers attached to the points indicate the number of leaves and leaf initials differentiated at the time of examination; brackets indicate that the state of the succeeding ridges of the young spike made it clear that no further leaves would be formed.

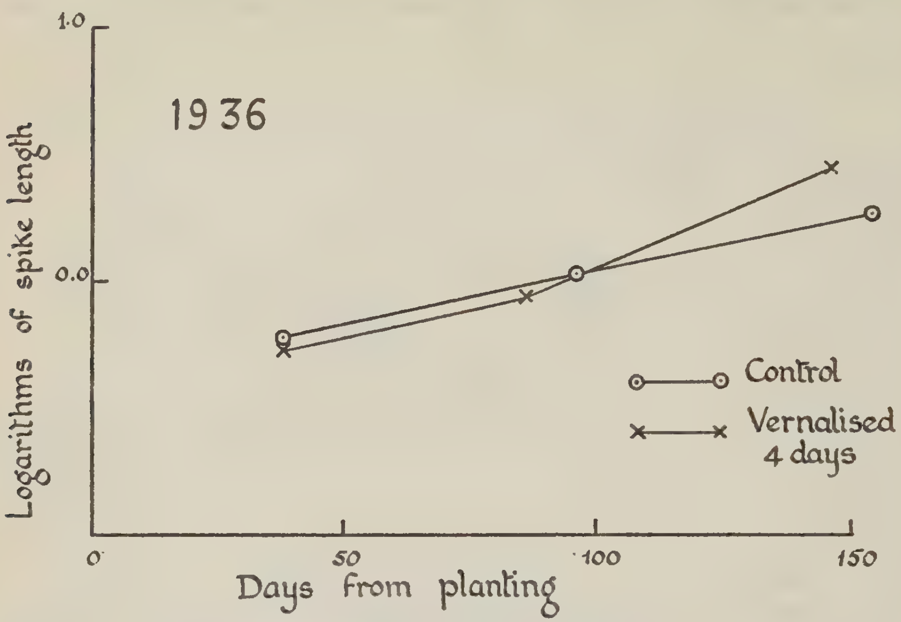


TEXT-FIG. 2. The effect of 4 days' low-temperature treatment on the growth-rate of the spike in winter rye, 1935.

There is, however, some evidence that a treatment as brief as four days has a slight vernalising effect which is too small to be apparent by this method of presenting the data. It was shown in an earlier paper that in unvernalised winter rye the relative rate of growth in length of the young spike is constant. Text-fig. 2, in which the logarithms of spike length are plotted against the time from planting, shows that this is true for the control series. Four days' chilling results in a sudden rise in the curve, and the time which elapses before this acceleration in growth begins varies inversely with the duration of the cold treatment. The experiment for the four days' exposure was repeated in 1936 and a similar result was observed, as shown in Text-fig. 3.

Table II presents the results of the experiment carried out in 1936.

The results of four days' treatment have already been described—in addition longer exposures were given in an attempt to obtain a maximal effect. The most rapid flowering follows treatment for fourteen weeks, with little change up to eighteen weeks. Longer exposures than this have increased the time to anthesis, but this effect is definitely due to advanced sowing date and consequent decreasing day-length at the time at which extension growth is due to begin. In the present year seedlings which were planted on May 26 after continuous exposure to 1·5° C. for twenty-three weeks attained anthesis in 48·2 days with a leaf number of 7·1; both figures are the mean of thirty



TEXT-FIG. 3. The effect of 4 days' low-temperature treatment on the growth-rate of the spike in winter rye, 1936.

TABLE II

Relation between Duration of Treatment and Length of Vegetative Period. 1936 Experiment

Planting date.	6/5.	6/5.	6/5.	6/5.	10/6.	17/6.	24/6.	1/7.	8/7.	15/7.	22/7.
Weeks of treatment	5	6	7	9	14	15	16	17	18	19	20
Days from planting to 1st anthesis	75	68	61	56	46	49	50	54	51	64	62
Days from sowing to 1st anthesis	110	110	110	119	144	154	162	173	177	197	202
Leaf number of earliest plant	11	11	10	9	7	9	8	7	8	10	8
% of plants earing	66	69	100	100	100	100	100	100	100	100	60

values. Spring rye planted on the same date produced 7·1 main axis leaves before flowering in 50·9 days (means of twenty-eight values). The slightly shorter vegetative period in this winter rye is probably due to the fact that some vegetative growth took place in the refrigerator during treatment.

With increasing exposures to low temperature both the vegetative period and the leaf number decrease progressively until the lowest possible leaf number (approximately seven) is attained. It is thus possible to assert that vernalisation of sufficient duration brings winter rye seedlings into the condition of spring rye both as regards leaf number and time of flowering. Vernalisation, however, cannot be carried beyond this stage, and continued treatment therefore increases the total time to anthesis from first breaking dormancy. This figure, as in the previous experiment, is constant for treatments from five to seven weeks. Nine and fourteen weeks' treatment do effect a further reduction in the time to anthesis from planting, but this reduction is less than the extra time spent under treatment, so that the total time from breaking dormancy is increased by such treatment.

VERNALISATION BY SHORT DAYS

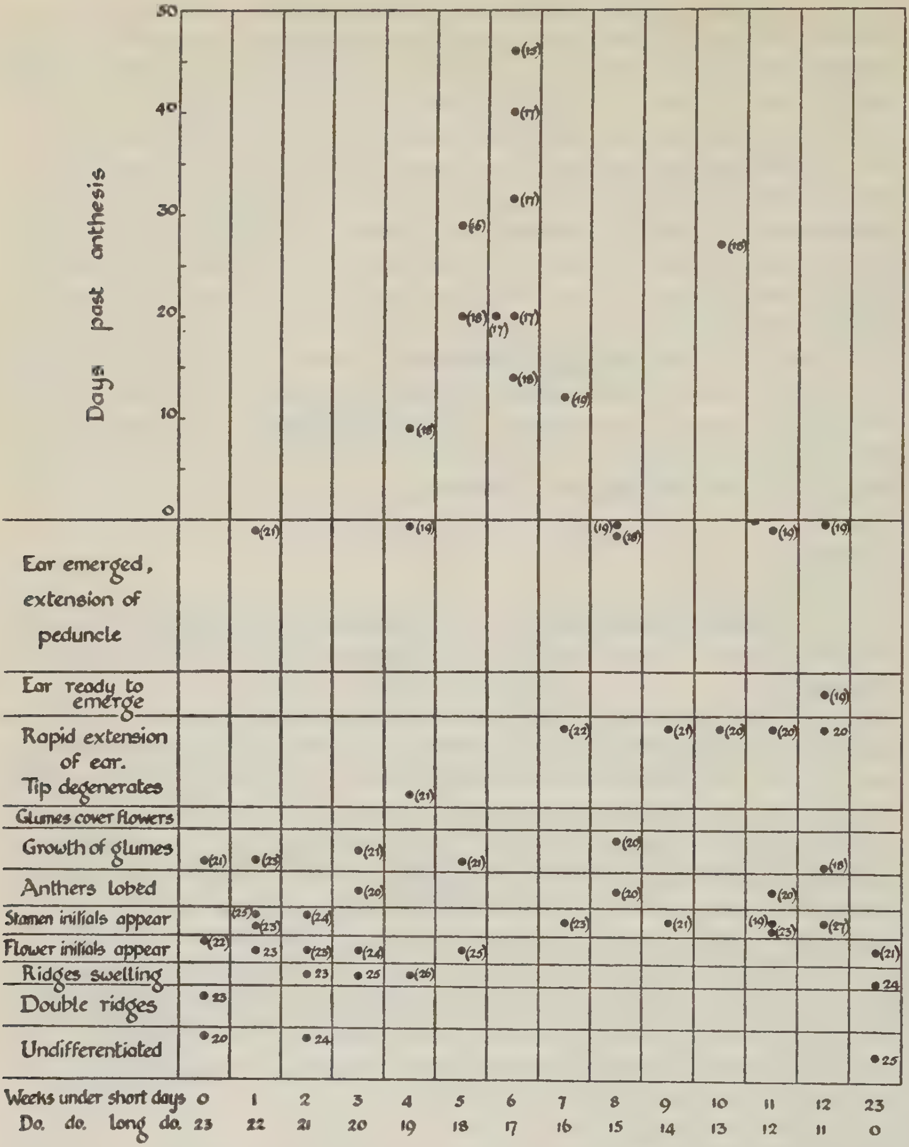
Experimental procedure.

Petkus winter rye was grown in pots when the days were naturally long, and during various preliminary periods was exposed to daylight for ten hours only each day.

In 1935 the plants were grown in sand culture (see footnote, p. 572) in eight 12″ pots, each pot containing initially ten plants. These were sown on May 14 and were exposed to short days for 1, 2, 3, 4, 6, 8, and 14 weeks respectively, a control pot being kept continuously in long days. At the end of each exposure to short days five plants from the pot being transferred were dissected in order to record the condition of the spike initial. The remaining plants were dissected at the end of the experiment, twenty-two weeks after planting. In 1936 a wider range of exposures to short-day conditions was used. Seed was sown in soil on May 6, again under a ten-hour day, and coleoptiles appeared on May 11, from which date the exposures to short day, ranging from one to twelve weeks, were measured. The condition of the plants twenty-three weeks after the experiment began was determined by dissection.

Experimental results.

Table III for 1935 gives the condition of the spike and leaf number at the time of change from short to long days, and also at the end of the experiment, while the results obtained in 1936 are shown in Text-fig. 4, which is constructed in the same manner as Text-fig. 1. It is very clear that in both years an exposure to short days of six weeks' duration was optimal for early flowering, and that more prolonged treatment tended to reverse this effect. In 1936 the accelerating effect of six weeks' treatment was much more marked.



Text-fig. 4. Diagram of winter rye grown in long days after different preliminary periods in short days, showing condition of growing-point, and examined 23 weeks after planting. Each plant examined is represented by a dot, and the numerals represent the leaf number at time of examination; when full complement of leaves is then present the number is enclosed in brackets.

The weekly dissections in 1935 showed that no morphological differentiation of flowers had occurred at the growing-point during this six weeks' period, but it can, however, be seen in Table III that shortly before the end of six weeks the number of lateral initials equivalent to the final leaf number had been differentiated so that subsequently formed initials developed as flowers. In 1936 it was determined that the first visible indication of flower differentiation (i.e. swelling of initials) occurred a few weeks later, i.e. after ten to eleven weeks.

TABLE III

Winter Rye. After-effect of Short Days, 1935

Treatment.	Weeks in short days.	0.	1.	2.	3.	4.	6.	8.	14.
Condition of main axis at time of change from short days to long days	Leaves	—	5·0	7·0	8·6	10·4	13·4	15·6	—
	Ridges	—	2·0	3·4	4·0	5·6	14·6	16·8	—
	Total lateral appendages	—	7·0	10·4	12·6	16·0	28·0	32·4	—
	Spike length (mm.)	—	0·17	0·23	0·34	0·42	0·74	0·97	—
Condition of main axis 22 weeks after planting	Further weeks in long days.	22.	21.	20.	19.	18.	16.	14.	8.
	Mean spike length	2·1	4·5	3·3	6·6	16·2	66·8	50·0	29·0
	Condition of most advanced spike in series	Near exsertion	Stamens differentiated	Exsertion	Awns growing	Near exsertion	Exsertion	Exsertion	Near exsertion
	Range of leaf numbers	20–5	23–7	23–6	24–4	20–5	18–24	19–24	19–22
	Mean final leaf number	—	25	24	24	23	20	21	21

Leaf number. In 1935 the smallest number of leaves was produced in plants which had received six weeks' short-day treatment, but the range of variation was so small that it seemed unjustifiable to attach very great significance to it (Table III). The results obtained in 1936 are shown graphically in Text-fig. 4. Here the effect of six weeks' treatment in the vegetative phase was much more marked: a much greater reduction in leaf number, i.e. to 16, occurred. Not only were more leaves than this formed after short-day exposures of less than the optimal, but also when the exposure was increased to seven weeks or more. The probable significance of this will be discussed in the next section.

DISCUSSION

Certain considerations put forward in an earlier paper (Purvis, 1934) are here elaborated further in the light of the new information presented above. In that paper the concept of 'ripeness to flower' suggested by Klebs was accepted, and was identified with the stage in development at which the 'minimal leaf number' had been laid down. This number was stated to be seven or eight in spring rye, while in vernalised winter rye it was thought to approximate twelve. In both the spring and winter varieties grown in short days it reached twenty-two, as it did also in unvernalised winter rye grown in long days.

These conclusions require modification in view of the results just presented. It is now seen that a quantitative relation exists between the duration of vernalisation treatment and the length of the vegetative phases, and the number twelve applies only to winter rye vernalised for the particular period (four weeks) used in those earlier experiments (cf. Table II).

The effect of low-temperature vernalisation on the number of leaves on the main axis and on flower initiation.

The shortest period used, namely, four days, was found to have an effect too small to manifest itself in accelerated flowering, but which was revealed by a study of the growth-rate of the apical meristem. Increased exposures proportionately curtailed the vegetative period until a maximum effect was attained, when with regard to leaf and flower production winter rye became the same in behaviour as spring rye.

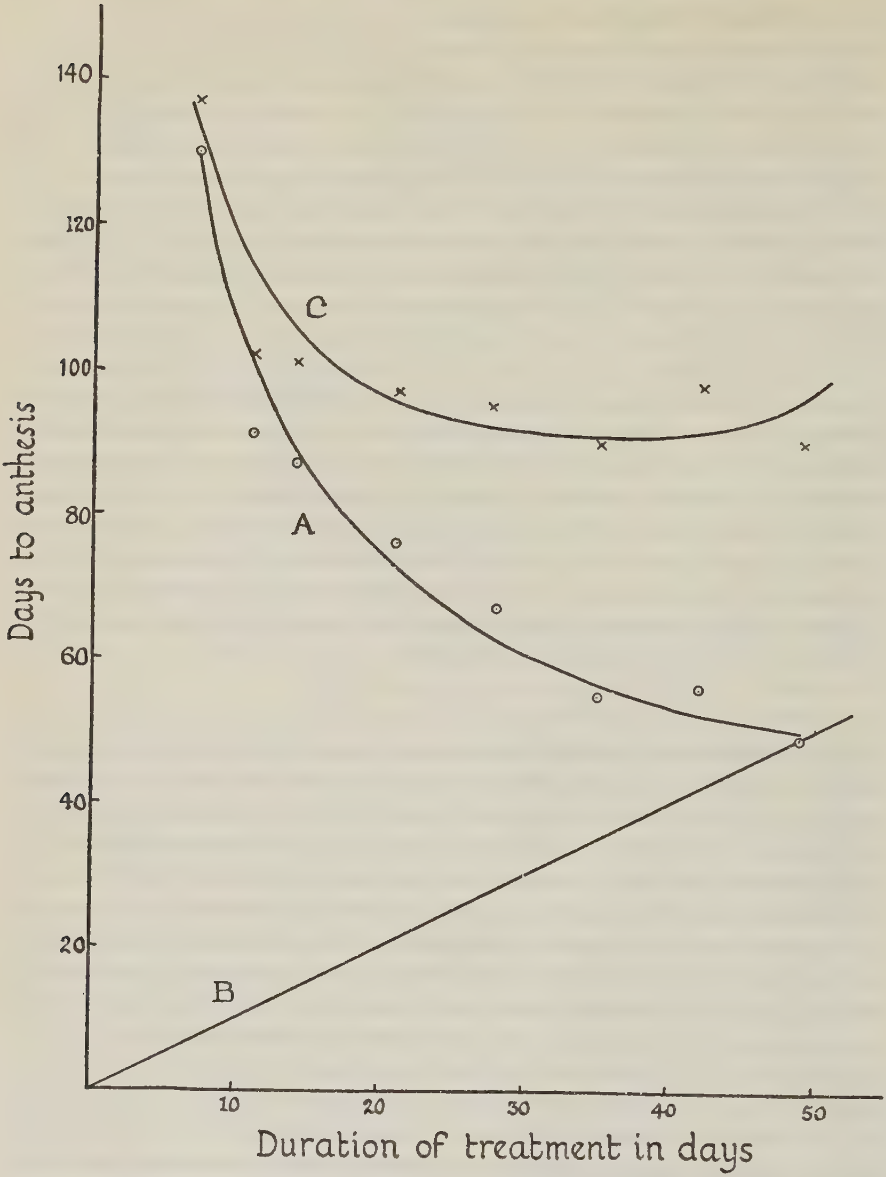
The foregoing data shows, therefore, that the 'minimal leaf number' in both spring and winter Petkus rye is the same (i.e. approximately seven). In the winter variety, however, the leaf number is reduced to this limit only after prolonged low-temperature treatment during germination, but in spring rye this number is not exceeded whatever may have been the temperature during germination. No treatment is yet known which can further reduce the leaf number in either variety. 'Ripeness to flower' may therefore be said to have been attained when seven leaf primordia are present. Reference to Table VIII on p. 936 of the communication cited above shows that this stage is reached very soon after germination. As the dormant embryo of rye already has four leaf primordia, and a fifth is differentiated during germination, irrespectively of temperature, the condition of ripeness to flower is reached some two weeks after germination, and the plant is then ready to proceed to flower differentiation under the appropriate external conditions.

Before passing to the vernalising effect of short days the bearing of these experiments on two points which may have been raised in the literature will be considered. It has been stated (Bull. 17) that prolonged periods of treatment lead to reversal of vernalisation, but Lojkin (1936), working on winter wheat, failed to confirm this. The results given on p. 576 show beyond all doubt that no such reversal occurs after nearly six months' treatment. The recorded cases of reversal refer, however, to red clover (Zerling and Cepikova, 1934) and cotton (Gavrilova, 1935), and there is no evidence of its occurrence in cereals. It is clearly unsafe at this stage to assume that 'vernalisation' in different classes of plants is one and the same process. Maximov (1934) has attributed to Vasiliev the statement that when the time of treatment is included in the length of the vegetative period this period is then no shorter than in unvernalised plants—in other words, the sole effect of chilling is to delay vegetative growth while permitting progress towards maturity. Even were such an explanation of vernalisation proved to be valid the practical value of the process would in no way be reduced, for approximately fifty days' progress towards maturity would still be made in embryos which show very little morphological development. There can, however, be little doubt that there is some specific cold effect other than this delay. In fact the English summary of Vasiliev's work indicates that he actually claimed only that the total time to flowering, including that of treatment, was constant over a limited range of durations and methods of treatment (cf. Vasiliev, 1934).

In Tables I and II (pp. 573 and 576) a similar result may be seen; there appears to be little variation in this *total* time with vernalisation treatments of three to seven weeks' duration, while beyond this range the total time increases. This admits of a simple explanation. The time from planting to flowering is a decrement curve approaching a minimum level with increasing exposures as shown in Text-fig. 5 (curve *A*). The time of treatment is increasing in a linear series (*B*): the total time being the summation of these two curves culminates in a rising straight line (curve *C*) parallel to *B*. Such a curve exhibits a range of values which are, relatively to the original axis, almost constant over a considerable period.

The vernalising effect of short days.

In the introductory section reference was made to the fact that after germination at 18° C. the rate at which new initials are laid down at the growing-point is greater in short than in long days. Without postulating any effect on leaf number, short days should therefore decrease the time taken to flower by accelerating the rate at which primordia are laid down so that the requisite leaf number is earlier attained. It has been demonstrated beyond doubt that leaf number is affected by the temperature of germination when the plants are grown in long days, but as regards a short-day régime, previous work (Purvis, 1934) has presented a very different picture. Both spring and winter rye, irrespective of temperature of germination, produce in short days rather more than twenty leaves on the main axis, and then proceed to the early stages of flower differentiation. There thus appears to be a maximal as well as a minimal leaf number, and when this maximal number is reached flower initiation begins, irrespective of temperature of germination or of day-length. It must, of course, be remembered that only one length of day has been systematically studied, namely, ten hours, and it is possible that with different light durations a different maximum would be attained. The point that requires emphasis here is that short days tend to increase leaf number. When the figures in Table III and Text-fig. 4 are examined it is seen, however, that in both 1935 and 1936 a *reduction* in leaf number has accompanied accelerated flowering as a result of a period of short days in the early stages of growth.



TEXT-FIG. 5. Time to anthesis from planting (A) and from beginning of treatment (C) after different periods of low-temperature treatment (curve B). For details see text.

This effect was not very marked in 1935, the lowest leaf number resulting from six weeks in short days being eighteen, and ranging up to twenty-four; in 1936, however, a range of fifteen to eighteen was obtained, indicating beyond reasonable doubt a reduction in leaf number. In both years the lowest minimum leaf number is associated with six weeks' treatment, the number being progressively reduced with treatments between four and six weeks in duration, but with exposures longer than six weeks an increase in leaf number again occurs. An examination of the meristem at the time of change from short to long days (Table III) discloses the fact that after six weeks in short days the total number of primordia already produced (i.e. total leaves+ridges) is twenty-eight. That is to say, that before the end of six weeks in short days the maximal leaf number has already been attained. It would appear, therefore, that the greatest effect of short days in reducing the leaf number occurs when the plants are removed to long days at a time when *a number of primordia corresponding with the maximal leaf number have been differentiated.* Further exposure to short days increases leaf number and thus delays ear emergence.

Theoretical considerations.

To account for the results obtained the following considerations are tentatively advanced. It has already been pointed out that the main axis produces a minimum number of leaves before flowering begins, and a maximal number beyond which lateral primordia are always differentiated as flower branches (spikelets) under any conditions. In Petkus spring and winter rye the minimal number is approximately seven, and the maximal, though less clearly defined, approximates to twenty-five. Thus each primordium between the eighth and twenty-fifth may produce either a leaf or a spikelet according, in both varieties, to the conditions of day-length and of temperature during germination in the winter variety. It must not, however, be supposed that either organ can be produced from the same cell-group in the primordium, and in this connexion the observed structure (Purvis, 1934) is of great interest. In barley and rye the initial ('ridge') presents at one stage in its development a double structure which Purvis interpreted as the initial of a bract with an axillary flower branch. A similar structure has been noted in wheat (Bonnett, 1936) and the same interpretation accepted.

If it were established that such 'double ridges' are found only above the seventh primordium it would clearly indicate that this development immediately follows the stage of 'ripeness to flower'; unfortunately no such observation has yet been made. It is clear, however, that the possibility of producing either a leaf or spikelet is inherent in such primordia, and observation has shown that if flower development proceeds the double nature of the ridge disappears, the upper part growing out as a spikelet while the lower part (bract) is completely arrested. In the previous paper (Purvis, 1934) it was stressed that the approach to an interpretation of the temperature after-effect and its interaction with day-length lay in the concept of 'failure to flower' rather than in a causation of flower initiation by the action of external factors. The same point of view has been further developed in relation to the general problems of photo-periodism (Gregory, 1936).

Regarding the problem under discussion from this direction, we may distinguish two aspects, namely, (1) an inherent tendency to flower production,

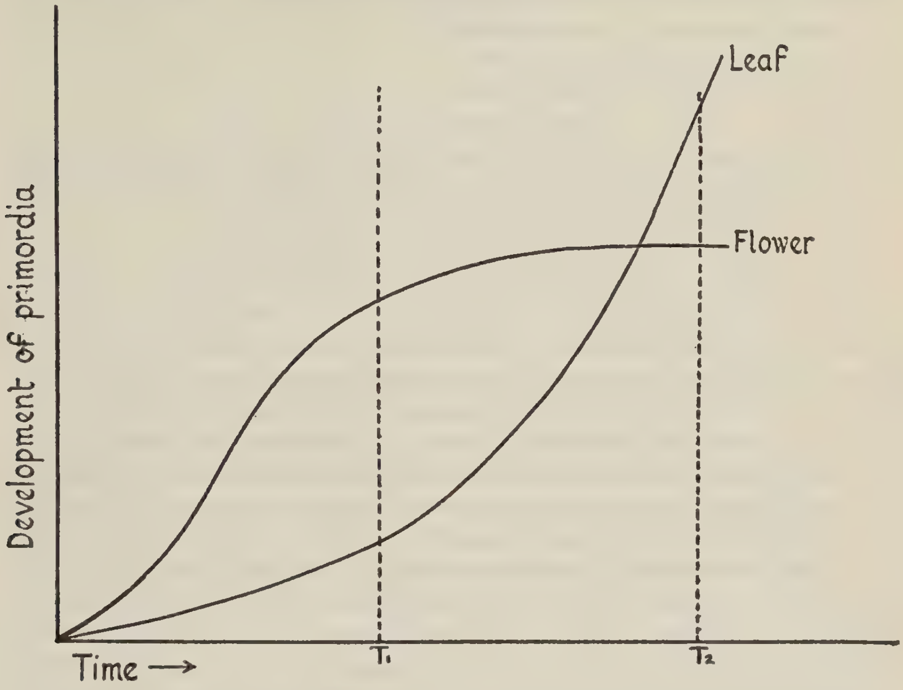
(2) factors controlled by the environment either directly as in the effect of day-length, or as an after-effect of the temperature of germination. At the present stage of the discussion the vague term 'tendency' will serve: later an attempt is made more rigidly to analyse this notion.

(1) The tendency to flower production clearly increases in the successive primordia after the first seven, in which it is apparently absent, since they always produce leaves, and rises in intensity to the twenty-fifth (approximately), which always develops into a spikelet, the intermediate primordia constituting a graded series. Further, this series (8th–25th primordia) represents an age succession, those at the apex being younger and therefore less advanced in development than the basal. The first appearance of flower differentiation should therefore occur somewhere within the series, and this agrees with observation on the young spike, and also with the well-known fact that anthesis begins some distance above the base of the ear. This order of succession in flowering is not uncommon in racemose inflorescences. Again, the few lowest spikelet groups in the ear are frequently sterile and undeveloped, while a well-marked ridge at the base indicates a strong tendency towards formation of a leaf-sheath.

(2) It would appear that certain conditions arrest development of one or other of the cell-groups in the double primordia leading to leaf or spikelet production. Something of these conditions is known. It is fairly general that short days lead to flower initiation (Gregory, 1936), though continuous short days may lead to arrest of development or even abscission of the flower bud as was noted in *Phaseolus multiflorus*. Winter rye germinated at 18° C. (unvernalised) is no exception to this rule, as short days here also accelerate the production of definite flower ridges (Purvis, 1934, Table IX). In continued short days, however, this development is arrested, and long days are definitely required for further differentiation into flowers in all primordia up to the twenty-fifth; beyond this stage even in continuous short days flowers are formed. In unvernalised winter rye, therefore, short days initiate flower production, but long days are required for further development, otherwise the flower primordia are arrested and the subtending leaf develops.

In the case of spring rye grown in long days, the eighth primordium produces a spikelet irrespective of temperature of germination, and in winter rye, as the period of exposure to low temperature at germination is increased, approximates more and more to this condition. The tendency to flower development increases continuously during vernalisation, but by keeping the plants in short days the eighth and all subsequent primordia to the twenty-fifth produce leaves in winter and spring varieties alike irrespective of temperature of germination. It appears, therefore, that either (1) short days arrest the flower primordium while the leaf primordium continues its growth unaffected, or (2) short-day conditions definitely favour the growth of the leaf primordium. As leaf-production rate continues quite unchanged in long or short days up to the sixth week from germination (Purvis, 1934, Table VII) independent of temperature of germination, the first assumption seems the more probable.

Conditions in the labile primordia. The conditions in the labile primordia then appear to be as follows: Under exposure to short days development of the flower is first favoured and then is rapidly arrested, whereas leaf development is unaffected. These conditions are represented in the following diagram.



TEXT-FIG. 6. Schematic diagram of growth relations in the labile primordia of winter rye in continuous short days. For details see text.

It is assumed that if the plant is removed from short to long days at time T_1 while flower development is in advance of leaf development a spikelet is formed and the leaf is arrested, while following later removal at time T_2, when the leaf has overtaken the flower in development, the spikelet is inhibited. It is known that the maximum duration concerned is in the neighbourhood of four weeks, since this period of time elapses from primordium formation to the definite appearance of the spikelet initial and disappearance of the 'double ridge'.

Conditions in spring rye show that flower initiation can here take place in long days, and on the assumptions made above the effect of short days must be due to arrested development. As winter rye passes from the unvernalised state to complete vernalisation earlier and earlier primordia, under long-day conditions, develop into spikelets. This must mean that the earlier primordia attain the capacity of developing into spikelets in long days, although the arrest by continuous short days will occur just as in spring rye.

If the foregoing considerations are at all correct it should happen sometimes that leaf development continues sufficiently far for a definite leafy bract of the lower spikelets to appear, and such ears have been observed. The following difficulty must, however, at once be met. The 'labile primordia' are differentiated during the second to sixth week from germination. The total number of primordia produced by unvernalised winter rye in short days (ten hours) are shown in the following Table IV.

TABLE IV

Total Number of Primordia produced by Unvernalised Winter Rye in Short Days

Weeks from germination.	Total primordia.
2	7
4	12
6	30
8	41

If then plants, after, say, four weeks, are removed from short to long days some of the labile primordia will have already been growing in short days and should have advanced in development beyond the subtending bract, while at the same time later-formed primordia will have been differentiated in long days and thus not have received an initial short-day period. It might be expected, therefore, that spikelets would be produced below, and then a reversion to leaf production take place. Such a return to vegetative growth has not been encountered in these experiments nor, as far as the authors are aware, has it ever been recorded. The assumptions made are therefore too simple.

The scheme put forward above takes no account of the qualitative differences in the series of labile primordia. Clearly each successive primordium from the eighth to twenty-fifth is less dependent on the effect of day-length for initiation and development of flowers. The lower members of the series in spring rye are already in a condition to produce flower initials even in long days, and vernalisation confers the same potency on the primordia of winter rye, if sufficiently prolonged. Therefore either some change in condition of the growing-point occurs during vernalisation or the accumulation of some definite substance has taken place. In either case the change involves an after-effect, since the primordia in which it is located are not yet present at the end of the low-temperature period when only five primordia are present at the growing-point. There is good reason to suppose that the whole plant and not the growing-point alone is changed by vernalisation, since the lateral branches (tillers) show the same flowering behaviour, although at the end of the period of vernalisation the majority of vegetative buds are not yet formed.

The whole situation is still obscure, though the following facts are established. (1) The hypothetical substance is not preformed in the endosperm and thence transferred to the embryo, as the embryo isolated from the seed behaves in a like manner (Gregory and Purvis, 1936); (2) the change is reversible by high temperature (Gregory and Purvis, 1936*b*). It appears possible that during vernalisation some precursor of a 'flower-forming' substance accumulates in the embryo. This substance may be supposed to be translocated and accumulated in all growing-points of the shoot, and when a critical concentration is reached induces flower initiation. The increasing rate of formation of primordia (cf. Table IV above) indicates that the substance increases autocatalytically. In spring rye approximately two weeks at normal summer temperatures after germination suffice to reach the critical level, while in winter rye unvernalised somewhat less than six weeks are required. The rate of formation is thus three times as rapid in spring as in unvernalised winter rye. (3) An oxidative reaction is involved in the formation as anaerobic conditions completely inhibit its production during vernalisation of winter rye (Gregory and Purvis, 1936*b*), and evidence is accumulating which suggests a partial destruction by anaerobiosis in spring rye. Finally (4) in spring rye the reaction is controlled by a genetic factor, since the F_1 hybrid between spring and winter rye shows the early flowering habit as dominant, and segregation in 3 : 1 ratio occurs in F_2 (unpublished data). The evidence on all these points will be presented in full in later communications.

During vernalisation the 'flower-forming' substance or its precursor increases in concentration so that the critical level is reached earlier, and thus spikelet initiation sets in earlier in the sequence of labile primordia, and a progressive reduction in leaf number occurs, finally reaching the minimum of 7 characteristic of spring rye. The progressive effect of low-temperature vernalisation can formally be accounted for on these lines.

The relations to day-length are more difficult to formulate. The outstanding problem here is related to the fact that in both spring and winter rye in continuous short days a maximum leaf number is found, which is approximately constant and independent of temperature of germination. In spring rye the 'flower-forming' substance is already present in high concentration, but only in long days do the early members of the series of labile primordia form spikelets. It appears, therefore, that two stages are involved in the process. A precursor depends for its formation on a genetic factor in spring rye and on low temperature of germination in winter rye. This precursor accumulates in all the growing-points, increasing autocatalytically. A second stage in the process involves reactions depending on day-length.

The relations may be schematically represented below.

$$\begin{tikzcd}
 & \text{E (leaf-promoting substance)} & & \\
\overset{\text{Precursor}}{\text{A}} \arrow[r] & \text{B} \arrow[u] \arrow[r, shift left, "\text{short day}"] & \text{C} \arrow[l, shift left] \arrow[r, "\text{long day}"] & \text{D (spikelet-promoting substance).}
\end{tikzcd}$$

The substance B is produced by an autocatalytic reaction from A as suggested above. The substance B under conditions of short day (by a reaction proceeding more rapidly in darkness) produces an intermediate substance C which is responsible for the early stages of flower initiation. Under short-day conditions C will tend to accumulate. The reaction B → C would appear to be reversible, the concentrations of B and C tending to an equilibrium. A secondary reaction to E proceeding at a slower rate leads to production of a specific leaf-forming substance favouring development of the leaf in the labile primordium. The reaction C → D, which occurs in the spikelet initial, is accelerated by light (possibly by a photocatalyst) and is irreversible; the substance D leads to further development of the spikelet.

The production of D or E will thus depend on the relative rates of the two alternative reaction-chains. In vernalised winter rye and in spring rye the concentration of B is high, and since the reaction B → E is independent of light the rate of formation of E will depend only on the concentration of B and on temperature.[1] The similar effects of high temperature and short days were shown by Eaton (1924) and confirmed in rye by the present authors. In short days, therefore, when the reaction C → D is slow, B will in the main be converted to E and the leaf primordium will develop, as is the case in winter and spring rye in continuous short days. In long days, however, spring rye produces spikelets while unvernalised winter rye produces leaves. This is due to the high concentration of B in spring rye even in the eighth primordium, whereas in unvernalised winter rye the concentration of B is low, due to low concentration of the precursor or the slow rate of the autocatalytic reaction A → B. The high concentration of B in spring and vernalised winter rye leads to formation of C even though the reaction proceeds slowly in long days, whereas rapid conversion of C → D, due to the catalytic effect of light, favours conversion of B by maintaining a low level of C. In spring rye, therefore, the concentration of D reaches the critical level for spikelet formation even in long days. Unvernalised winter rye has low concentration of B and therefore the production of E and D proceeds slowly: in consequence, in long days the concentration of D fails to reach a critical level in the early primordia, and the bract develops. In vernalised winter rye in long days, and in the later primordia of unvernalised plants also, spikelet production takes place, due to the accumulating concentration of B resulting by autocatalysis from A.

In unvernalised winter rye in short days the reaction B → C proceeds rapidly so that spikelet initiation begins, and therefore if a preliminary period of short days is followed by long days spikelet formation proceeds even in the earlier labile primordia. This is the process of short-day vernalisation described above. In continuous short days, however, the reaction C → D is slow, the accumulation of C tends to reverse the previous reaction and favours the secondary reaction B → E; thus spikelet primordia are inhibited as D fails to reach the critical level, while leaf formation rapidly proceeds. In the series of labile primordia (8th–25th) the level of concentration of B rises, and thus of D also, so that eventually even in short days the critical level is attained and spikelets develop.

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[1] The interaction of temperature and day-length on flower production is discussed by Steinberg and Garner (1936).

Regarding now the process of short-day vernalisation: this, as is shown above, results in a reduction in leaf number after a preliminary short-day régime of four to six weeks, with an increase in leaf number if the period is extended beyond six weeks. During this period the full number of labile primordia is laid down. Concomitantly the concentration of B is rising, so that the level of C also increases throughout the series of labile primordia. On removal to long days some of the primordia have, therefore, sufficient of C present to produce the critical level of D as soon as the photocatalytic reaction C → D takes place. Some of the primordia which in continuous short days would eventually have produced leaves thus now produce spikelets, and the leaf number is reduced. These primordia, on the assumptions made, will be the later members of the series. If the short-day régime is prolonged beyond six weeks primordia are then being formed which in any case would produce spikelets, whereas in the earlier primordia leaf growth has proceeded and reduced the level of B to such an extent that even in long days the critical level of D is not reached. The considerations outlined above thus account for a fall and subsequent rise in leaf number as the preliminary short-day period is extended. With regard to the schema put forward, its hypothetical nature is very evident. So far as the irreversible stage C → D is concerned, it is possible that the critical event is the differentiation of the megaspore mother cell, and evidence on this point will be sought. The postulation of two reactions, one dependent mainly on temperature of germination, the other on a photocatalytic process, resembles in some respects the suggested theory of Lysenko, though the present hypothesis differs in that the stages are not regarded as absolutely distinct; further it has been clearly demonstrated, Lysenko's 'thermo stage' is not obligatory for the initiation of flowering, but is of a quantitative and not purely qualitative nature.

The experiments were performed at the Chelsea Physic Garden, and the authors here record their thanks to the staff for the very necessary work of moving the plant cultures in and out of the dark shed.

SUMMARY

1. The effects of varying preliminary periods of low temperature and short days on the subsequent development of winter rye are described.

2. Both treatments result in acceleration of flowering.

3. With low-temperature treatment four days' exposure leads to a definite response, and the magnitude of the response increases with the duration of treatment up to a limit of fourteen weeks, after which winter rye is indistinguishable from spring rye in its flowering behaviour. With increasing

exposure to low temperature the leaf number in winter rye is reduced from approximately twenty-five to seven.

4. With preliminary short days (ten hours) the magnitude of the effect on flowering increases with length of treatment up to six weeks, but longer exposures retard flowering. Reduction of leaf number to a minimum of sixteen occurs after six weeks' treatment with an increase after longer treatment.

5. In both spring and winter rye there is a minimum leaf number, seven, which cannot be further reduced, and a maximum, twenty-five, which cannot be further increased.

6. The primordia between the eighth and twenty-fifth are indeterminate and can produce either leaf or spikelet: this is related to the structure of these initials, which are double, consisting of flowering branch (spikelet) with a subtending bract, either of which may be inhibited according to the length of day or temperature of germination.

7. A scheme is presented, postulating leaf and flower-forming substances whose rates of formation are controlled by external factors, which fits adequately the experimental results.

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DESCRIPTION OF PLATE XXII

Illustrating Dr. Purvis's and Dr. Gregory's paper on 'A Comparative Study of Vernalization of Winter Rye by Low Temperature and by Short Days'

Fig. 1. Vernalisation by low temperature. Winter rye plants 14 weeks after planting out, following vernalisation for different periods. The numerals represent number of days at 1° C.

Fig. 2. Vernalisation by short days. Winter rye plants grown in long days after preliminary periods in short days (10 hours); the length of the period is indicated by a numeral which represents weeks. Photograph taken 19 weeks after planting out.

Mycorrhiza in Rhododendron

BY

H. D. GORDON, Ph.D.

(*From the Department of Mycology, University of Edinburgh*)

With Plate XXIII

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INTRODUCTION

IT has long been known that the roots of Rhododendron, in common with those of the rest of the Ericaceae, contain an endophytic fungus, but the genus has received very much less attention from this point of view than several others, notably Calluna and Vaccinium. Yet it is a genus of rather peculiar importance in any consideration of the significance of mycotrophy in this family, for it includes a group of species, mainly from the Yunnan area, which grow naturally on limestone, a very exceptional habitat for Ericaceous plants. Some of these species have been experimentally cultivated on calcareous soils in this country, as on the chalk hills of east Berkshire (Grove, 1916), and have been found to survive and flourish under conditions which would be entirely unsuitable for the majority of Ericaceae. Attempts have been made to explain these facts on the basis of the mycotrophic habit, including the speculative suggestion that, when the plants are growing on lime, the fungus migrates from the roots to the under surface of the leaves where it occupies a highly favourable place for the fixation of atmospheric nitrogen. This is but one expression of an idea that has gained some currency, namely, that these species of Rhododendron owe their tolerance of lime in some way to their mycotrophic habit.

But the type of mycorrhiza found in these lime-tolerating species, at least when cultivated in the usual way on non-calcareous soils in this

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country, is quite similar to that of the other species of the genus, and of the majority of other genera in the family. It is of the same type which has been described, in particular, for Calluna and Vaccinium. Some time ago Rayner (1913 and later papers) suggested that there might possibly be a connexion between mycotrophy and the calcifuge habit in the Ericaceae. The fact that lime-tolerating and lime-shy members of the family, at least when growing under similar conditions, form the same type of mycorrhiza emphasizes the need for great caution in relating the opposing peculiarities of either group to a factor which is common to both. Information as to the presence or absence of the endophyte, and its state of development if present, in the roots of lime-tolerating Rhododendrons when actually growing on their native limestone is greatly to be desired.

Rayner (1915), in her original paper on 'Obligate Symbiosis in *Calluna vulgaris*', included Rhododendron species amongst those in which she had observed hyphae in the ovary, and to which it was inferred that the distribution of the fungus throughout the shoot, then described for Calluna, would also apply. More recently Freisleben (1935) included four species of Rhododendron amongst a large number of Ericaceae which he investigated, and though his results were by no means in agreement with those of Rayner, he also found that Rhododendron conformed to the general type. That has likewise been the experience of the writer, so that the normal mycorrhiza of Rhododendron may be quite briefly described.

There is no dense, external sheath of hyphae, as in most examples of tree mycorrhiza, nor do the roots assume a stunted 'coralloid' appearance. Rather are they long and thread-like, and the surface, which is devoid of root-hairs, presents a glistening white appearance to the naked eye . While this surface is freely exposed to view, microscopic examination shows that fairly abundant hyphae do nevertheless ramify over it. They are slender, sparsely septate, and give off branches which penetrate the root. These branches may enter the large epidermal cells directly, through their outer walls, or may force a way between these cells and enter through their lateral walls. Within each cell they form a more or less closely interwoven coil of very slender hyphae, generally almost filling the cell. Hyphae may be observed to pass from one such cell to another, but the fungus does not seem to spread very far in this way from a point of entry; the majority of the epidermal cells show direct connexions between the internal and external hyphae, and have probably been independently infected from the superficial mycelium. While these hyphal complexes in the epidermis are by far the most conspicuous evidence of the presence of the fungus, hyphae may also be found, to a less degree, in the underlying cells of the cortex, which is a very much reduced tissue in the roots of the Ericaceae. The typical, naturally developed mycorrhiza described above is very uniform throughout the major part of the family, and previous descriptions are in agreement on its main features.

REVIEW OF PREVIOUS WORK

In 1915 Rayner, as a result of her work on Calluna, subsequently extended to Vaccinium, put forward a conception of the mycotrophic relations of the Ericaceae which has been a subject of controversy ever since. In particular the following three opinions expressed by Rayner have been contradicted and reaffirmed:

1. The endophytic fungus is not confined to the roots, but penetrates to all parts of the plant.
2. (which depends on 1). The fungus enters the ovary and infects the seeds, at least as far as the testa, so that it is dispersed with them and infects the seedlings on germination; in this way infection is assured, and may be described as 'cyclic'.
3. Infection by the fungus is necessary for the development of the seedlings. In its absence they cease growth after expanding their cotyledons, and in particular are totally unable to form roots.

The first two may be considered together. The presence of hyphae in the sub-aerial parts of the plant and their dispersal with the seed have been denied by Christoph (1921) and Freisleben (1934). The latter carried out very extensive investigations on many genera and species, especially on Vaccinium, the genus for which Rayner (1929*a*) had recorded the most deep-seated seed infection. It is clear, therefore, that there has been a marked divergence of opinion as to the facts of the case; it is not simply a question of discrepancy in the results reported for different genera. As against these denials, a single apparent confirmation is afforded by the papers of Addoms and Mounce (1931, 1932) working in America on *Vaccinium macrocarpon*. The writer must agree with Freisleben (1934) that it is doubtful whether the structures figured do, in fact, represent hyphae. In any case, they are certainly quite different from the inconspicuous shoot infection described by Rayner, and cannot, therefore, be regarded as a confirmation of her work.

The third controversial tenet, that of 'obligate symbiosis', or the inability of the seedlings to form roots in the absence of the fungus, has been contradicted particularly by Knudson (1929), who grew pure-culture seedlings of Calluna on various agar media, and published photographs of such seedlings with well-developed root-systems. The burden of Rayner's reply (1929*b*) was that such seedlings probably contained the endophyte which, being very scanty and attenuated under such conditions, had been overlooked. Knudson (1933) met the various criticisms of his previous work, and considerably strengthened his claim that Calluna seedlings can develop and form roots under sterile conditions.

An interesting series of experiments carried out by Freisleben (1934–5) led him to the conclusion that Ericaceous seedlings fail to form roots in pure culture on ordinary agar media, and also on a medium of peat and sand used by him, so that up to this point he is in agreement with Rayner, but that their further development and the formation of roots can be promoted by any fungus that does not behave as a parasite, so that this favourable influence is not a specific attribute of the endophyte. For example, contamination of the substrate by Penicillium and other common saprophytes was sufficient to ensure successful development of the seedlings, although the hyphae of these fungi did not penetrate the roots. This is quite a different result from that of Knudson, whose seedlings formed roots in the absence of any micro-organisms whatever.

Further light is shed on these questions by the experiments of Molliard (1934), who devised an ingenious medium consisting of cotton-wool soaked with an aqueous extract of peaty soil, the whole being sterilized. In this way the seedlings were supplied with a soil solution suitable for their growth, while the cotton-wool was easily penetrated by their roots, thus avoiding a very probable disadvantage of agar or of sterilized peat, which forms a densely coagulated mass. On this substratum development and root-formation were successful so long as moisture was adequate, thus confirming the opinion of Knudson, but in the higher and drier parts of the same tubes the seedlings failed to form roots and displayed symptoms of arrested development similar to those recorded in Rayner's pure cultures. This strongly suggests that such cases of inhibition are to be attributed to cultural conditions unfavourable for growth, and not to the absence of the endophyte or even of fungi generally. It is worthy of mention, in passing, that McLennan (1935) reached similar conclusions in the case of *Epacris impressa*, a representative of the Epacridaceae which take the place of Ericaceae in Australia, and normally possess a mycorrhiza of the same type.

The object of the present work has been to apply similar inquiries to Rhododendron, and to offer answers, in the case of this genus, to these controversial questions in the mycotrophy of the Ericaceae.

Materials and Methods

Some of the methods used are necessarily incorporated in the accounts of the particular experiments to which they apply. It will be convenient, however, to group here a few routine methods which were used repeatedly, and to which reference will then be made briefly in the later descriptions.

Aseptic extraction of seeds.

In investigating the question of seed infection within the parent ovary, it is of prime importance to obtain seeds from such ovaries without exposing them to any risk of subsequent contamination, and without disinfection or other treatment which might destroy any fungus already present. This is done by superficially disinfecting the capsules, dissecting them, and removing the seeds with aseptic precautions to a sterile culture vessel of some kind. The capsules must be carefully examined under a lens to ensure that no slight dehiscence has occurred, which might have afforded an entry to micro-organisms.

Several methods of sterilizing the surface of the capsules were tried. The easiest and most rapid consisted of immersing the capsule for a moment in methylated spirit, which was then set alight; with the relatively large and thick-walled capsules of many species this can be done without injury to the seeds, but it was found less satisfactory in eliminating contamination than those methods in which mercuric chloride is the sterilizing agent.

One such method is that described by Norton and Chen (1920) for the superficial sterilization of seeds. The seeds, or in this case capsules, are first thoroughly soaked in water (10–12 hours). The object of this is to protect the internal tissues by preventing excessive penetration by the sterilizer, which might occur if the seeds or capsules were dry. They are then shaken for three to five minutes in a sterilized test-tube with alcoholic corrosive sublimate (2 gm. to 1,000 c.c. of 50 per cent. alcohol). This is followed by rinsing in 93 per cent. alcohol, and in three changes of sterile water. This method was found to be satisfactory, but not necessary, for the present purpose.

In most cases the capsules were simply immersed in mercuric chloride solution, in which they were vigorously shaken to remove air bubbles and ensure thorough wetting, and then rinsed in three changes of sterile water. Concentrations from 0·1 to 1·0 per cent. of mercuric chloride were used, and it was found that in the case of Rhododendron capsules there is a large degree of latitude, and sterilization may be made amply adequate without danger to the seeds, as the interior of the capsule is well protected by the hard inner pericarp.

After any such treatment the capsule was quickly dissected with flamed instruments, and the seeds removed with a flamed inoculating loop to the sterilized medium on which they were to germinate. In most cases this was 2·5 per cent. malt agar, which readily revealed the occasional contaminations which, not unnaturally, occurred. In some cases, seeds extracted in this manner were sown on other media, to which reference will be made in the appropriate parts of the text.

Disinfection of seeds.

To study the growth of 'pure culture' seedlings, i.e. seedlings grown in the absence of the endophyte as well as of other micro-organisms, without prejudging the question of seed infection within the parent ovary, it is necessary to subject seeds to a disinfecting or sterilizing process to kill any fungus that may be present on or in them. Such treatment was also necessary in all cases where the seeds had not been aseptically extracted from the ovary—for instance, where they had been collected on dehiscence of the capsules and stored in seed-packets.

Immersion of seeds in 1 per cent. mercuric chloride for three minutes, the treatment used by Rayner (1915, 1922) for Calluna, was sufficient to kill the Rhododendron seeds; Rayner states that the margin of safety with this method is a very narrow one. More satisfactory results were obtained in the present work by using a 0·1 per cent. solution of mercuric chloride; immersion in this solution for three to six minutes, followed by rinsing in three changes of sterile water, was sufficient to sterilize the seeds without preventing germination.

It has been found, however, in common with the experience of most other workers in this field, that calcium hypochlorite is a more suitable sterilizing agent for regular use than mercuric chloride. It is completely effective, but free from the danger of killing the seeds, and the absence of any necessity of rinsing reduces the risk of contamination after sterilization. In some of the earlier experiments controlled concentrations of hypochlorite solution were used, but this was found to be unnecessary as a saturated solution was perfectly safe. The solution was always prepared fresh as required by shaking distilled water in a test-tube with an excess of calcium hypochlorite for at least five minutes, and filtering.

As Rhododendron seeds tend to float on the sterilizing solution, and so remain dry on their upper surface, they were first of all thoroughly soaked in water, in which their buoyancy was overcome by shaking, centrifugalizing, or 'air-pumping', or by a combination of these methods. This is most essential where it is required that the sterilizer should act for a stated short time, as when using mercuric chloride, but even with the greater latitude permitted by calcium hypochlorite it is desirable, as ensuring that no part of the seed is protected by air-bubbles from the action of the sterilizer.

Seeds soaked in this way were then covered with a saturated solution of calcium hypochlorite, prepared as described above, for widely varying periods of time, and transferred directly, without any rinsing, to the medium on which they were to germinate, generally malt agar, the transference being carried out with a flamed inoculating loop. A little of the hypochlorite solution is, of course, transferred with the seeds, and indeed they are permeated with it. This greatly assists in the preservation of sterility, the chlorine being gradually dissipated afterwards. It follows that the exact duration of immersion in the sterilizing solution before transference can have but little significance, as the seed is still subject to the action of the sterilizer after transference, and this influence ceases gradually, not at a precise instant. It is not, therefore, surprising to find that immersion for so short a time as four minutes before transference was sufficient to ensure sterility, while periods up to eighty minutes were used without any sign of harm to the seeds or the resulting seedlings. After these preliminary trials the immersion was generally kept between half an hour and one hour. Within half an hour the seeds had been thoroughly permeated by the sterilizing solution; they had been bleached almost pure white and were semi-transparent. Thus any internal fungus was certainly fully exposed to the action of a sterilizing solution which proved most effective in preventing any fungal growth on the malt-agar slopes to which the majority of such seeds were first transferred.

This method proved quite as effective as mercuric chloride treatment in the production of pure culture seedlings; it was much more effective than the latter agent in preserving the purity of the cultures, several hundreds of seeds being germinated on dozens of malt agar slopes without any contaminations occurring; it was much less liable than mercuric chloride to damage the embryo. But still another advantage of calcium hypochlorite falls to be recorded; it had a most marked beneficial effect on the germination of the seeds, resulting both in acceleration and in an increased percentage of germination. The latter effect is illustrated by the results of an experiment in which a large number of seeds of *Rhododendron decorum* treated with calcium hypochlorite for about half an hour, and a comparable batch which had simply been soaked and washed in sterile water, were germinated on filter-paper moistened with sterile water. The cultures containing unsterilized seeds became contaminated, but the development of the contaminant fungi on the filter-paper was sufficiently slight to permit of the following count being made after eleven weeks. As no new seedlings had appeared for several weeks prior to this date it seems improbable that the remaining seeds would have germinated at any later date.

	Number of seeds sown on Sept. 20, 1935.	Results on Dec. 8, 1935 Germinated.	Percentage germination.
Unsterilized seeds	71	46	65
Seeds sterilized with CaClOCl	84	78	93

Freisleben (1934) recorded a similar effect of calcium hypochlorite on the germination of Vaccinium seeds, though he did not observe it in the case of other genera of the Ericaceae.

Media.

The majority of seeds used for pure culture experiments after disinfection, as well as those aseptically extracted from the capsules, were germinated on 2·5 per cent. malt extract agar, which is a very favourable medium for the growth of most fungi, and a useful one for checking sterility. It was used also for verifying the sterility of those media, such as sand, in which fungi might readily grow without becoming apparent. A little of the sand, or other friable substratum, was transferred with a flamed inoculating loop to malt agar slopes, on which, if a fungus were present, it developed freely.

For continued growth of some seedlings the standard malt extract agar was diluted with approximately its own volume of sterile water, and again sterilized. This medium, in which the concentration of malt extract was approximately 1·25 per cent., was allowed to set with the tubes vertical, so that a deep column of agar with the minimum surface was obtained. Such cultures did not readily dry out, and the seedlings grew much more successfully in this diluted agar than in the original 2·5 per cent. medium.

Several agar media were used in a few cases each, but the only other which was used in considerable quantity was Knudson's Solution B. The formula is as follows (Knudson, 1933, p. 118):

$Ca(NO_3)_2 6H_2O$	1·0 gramme
$(NH_4)_2SO_4$	0·5 ,,
KH_2PO_4	0·25 ,,
$MgSO_4 7H_2O$	0·25 ,,
$FePO_4$	0·002 ,,
Agar	17·50 ,,
Distilled water	1 litre.

A medium such as this aims at supplying a full nutrient solution of salts suitable for the growth of green plants, instead of the organic nutriment required in media intended for the culture of micro-organisms.

All these media are subject to the disadvantage that the slender roots of Ericaceous seedlings do not readily penetrate solidified agar. They proved capable of growing through the diluted but still solid malt agar mentioned above, but the rather unfavourable physical character of such substrata was emphasized by the frequency with which roots remained above the surface of the medium, sometimes becoming arched in their growth as though prevented by the smooth, unbroken surface from extending apically in the normal manner. To obviate this difficulty a number of seedlings were grown in clean sand irrigated with Knudson's Solution B, without agar. The sand was distributed in conical flasks, and after the solution had been added they were plugged with cotton-wool and autoclaved. Such a medium offered good physical conditions for rooting, combined with the suitable chemical composition of the culture solution.

Somewhat similar in principle is the cotton-wool medium devised by Molliard (1934), briefly referred to in the survey of previous work. A pad of absorbent cotton-wool was arranged on one side of a large test-tube. The liquid phase in this case was an aqueous extract of a peaty potting soil suitable for the growth of Rhododendron. Equal volumes of soil and distilled water were mixed and allowed to stand for three days; then the liquid was decanted, filtered, and distributed in the tubes so that the cotton-wool was saturated, and a reservoir of free liquid remained in the bottom of the tube. The tubes were then plugged and autoclaved.

Finally, use was made of the peat-sand mixture described by Freisleben (1933, 1934, 1935). A mixture of powdered peat and clean sand was distributed to a depth of about 2 cm. in small Erlenmeyer flasks, mostly of 125 c.c. capacity, and rather oversaturated with distilled water. The flasks were then plugged and sterilized by steaming at 100° C. for forty-five minutes on each of three consecutive days. The peat swelled up and absorbed the excess water, once experience had shown the correct proportions to use, so that a thoroughly moist but porous medium resulted.

Staining.

The hyphae of the endophyte are most readily observed by the use of some form of the cotton blue staining method; this has been the experience also of previous workers in this field. The hyphae in infected roots could mostly be identified without staining, but in all cases where their presence was in doubt the roots or other members were stained with cotton blue. Thus it is to be understood that whenever a plant or member is recorded as free from infection this result is based on careful examination after staining in cotton blue, as well as, in most cases, a preliminary examination in the unstained condition. Best results were obtained by immersing the member in lactic acid or lactophenol until thoroughly cleared, then overstaining in a concentrated solution of cotton blue in lactic acid and differentiating to the required degree in lactophenol, in which examination was carried out. Preparations permanently mounted in Canada balsam were never so satisfactory as those mounted in lactic acid or lactophenol.

The Question of Shoot and Seed Infection

In all normally grown plants examined, both adult plants and seedlings which have been cultivated in potting soil during these experiments, the hyphal complexes in the root cells were easily recognized without staining, but staining in cotton blue made the details of infection still more evident. Small seedlings were frequently stained and mounted entire in lactic acid or lactophenol; examined in this way the hyphae in the root were extremely obvious, yet none could at any time be recognized in the hypocotyl or higher parts of the young shoot. In the case of adult plants too, sections were cut from stems, leaves, pedicels, ovaries, and ripening capsules, while ripe and unripe seeds were sectioned, and also stained and mounted entire. But though the hyphae in the roots could always be seen with ease, consistently negative results were obtained by detailed examination of the other parts.

An attempt was made to isolate in culture any fungus that might be present in the tissues of the shoot, despite the above negative results. The material was obtained from a pot of *Rhododendron ambiguum* seedlings which had been grown in ordinary potting soil to study the course of normal infection. This isolation experiment was carried out in April 1936, when the seedlings were almost two years old. The roots showed copious and typical mycorrhiza, and this was verified in the case of the seedlings raised for use. Small pieces of the various shoot members were thoroughly washed in water and then superficially sterilized, some in 1 per cent. mercuric chloride for one and a half to two and a half minutes, others in calcium hypochlorite for fifteen minutes. In both cases the pieces were then thoroughly rinsed in sterile water and quickly cut up with flamed instruments. In the case of cylindrical parts—stems, petioles, and leaf midrib—both ends were cut off, and a thin layer of tissue removed from the surface, while pieces of leaf lamina were trimmed all round, the object being to expose fresh surfaces where living hyphae might be present. The pieces were then transferred to malt and oat agar media, but no fungus grew from any of them. Such an experiment is not in itself sufficient proof of the absence of living hyphae from these parts of the plant, for it is notorious that attempts to isolate Ericaceous endophytes, even from roots known to be heavily infected, have only succeeded in a very few cases. It is also possible that any internal hyphae had been killed by the sterilizers, but it seems improbable that these had penetrated throughout the tissues before they were removed with sterile water. It would be inadmissible to draw a conclusion from this experiment alone, but it is in accord with that obtained by microscopic examination, namely, that the endophyte of Rhododendron is confined to the roots.

Evidence on the question of seed infection must be sought by germinating seeds aseptically removed from the capsules by the method already described. Applying this method to Calluna and Vaccinium, Rayner (1915, 1929*a*) reported that fine hyphae grew out from the testa and infected the primary root. No such hyphae have been observed in the present work on Rhododendron. Aseptically extracted seeds were germinated on filter-paper moistened with sterile water, and examined both stained and unstained at various stages of growth, without revealing any sign of the presence of hyphae, nor did any appear when such seeds were germinated on malt agar. It seems, therefore, that the endophyte is not seed-borne, but must infect seedlings afresh from the soil under normal conditions.

THE COURSE OF NORMAL INFECTION IN SOIL

If infection normally takes place from the soil after germination, it becomes a matter of some interest to follow the course of this infection, and to find what relation, if any, it bears to root development. Accordingly, in the early summer of 1934, a pot of ordinary potting soil was sown with seeds of *Rhododendron ambiguum* (1933 crop) from a seed-packet. Neither soil nor seed was subjected to any process of sterilization; the pot was left in the seed-pit and its treatment was similar in all respects to that of other pots of Rhododendron seedlings being grown in the course of regular horticultural practice. At intervals from germination onwards typical seedlings were removed and examined for the beginnings of infection. The seedlings were washed thoroughly and mounted entire in water, then stained in cotton blue and the examination continued, still keeping the seedlings entire. The results of examination at various dates are shown in tabular form below.

Infection of R. ambiguum *in potting soil, 1934*

May 31.	June 21.	June 29.	July 16.	Aug. 15.	Sept. 28.	Oct. 26.
Seed sown	–	–	–	+	+	+
					–	+

As in all such pot cultures, the seedlings showed considerable differences *inter se* in their respective growth rates. On September 28, more than a month after mycorrhiza had first been recognized, four seedlings were selected, ranging from one of the largest in the pot to one of the smallest. The root-systems of the two largest were copiously branched, and normal mycorrhiza was observed. The root-system of the third seedling was elongated, but rather sparingly branched, and no infection was present. The smallest seedling had a proportionately small root-system, though growth and branching had taken place; here again the cells were quite uninfected. Infection, then, had not been by any means simultaneous throughout the pot; large seedlings seemed to be infected first and, if there is a causal connexion between the two, then the size, or stage of development, must have conditioned infection, not vice versa, for the individual differences in development amongst the seedlings were present before infection could be recognized in any of them. On October 26 five seedlings were similarly selected, ranging from one of the largest to one of the smallest, but in this case mycorrhiza had been formed even by the smallest seedling examined, though infected cells were still much fewer than in the larger seedlings.

The most interesting, and rather unexpected result of this investigation is the complete absence of infection for more than six weeks after sowing. By June 21 root growth was visible; by July 16 several of the seedlings examined had well-developed, branched systems of slender white roots, and had produced their first epicotylar leaf, but neither in root nor shoot were any internal hyphae to be seen. The seedlings examined on August 15 and later dates revealed the early stages of mycorrhiza development; infected cells appeared sporadically in the roots, sometimes singly, sometimes in little groups. Several such infected regions might occur along a single root, separated by regions where the cells were still uninfected, showing that infection was taking place from the soil more or less simultaneously at many points and was not spreading from a single centre, nor from main root to laterals as these emerged. It was noted here, as in the roots of older plants, and as mentioned in the earlier description, that almost every infected epidermal cell showed an independent connexion with the superficial mycelium.

In all cases in this experiment with *R. ambiguum* and in pot cultures of several other species including *R. decorum* and *R. ferrugineum*, infection was confined to mature, vacuolated cells. Rayner (1915) has described hyphae from the testa of Calluna as infecting the tip of the root, as well as other parts. In all the roots examined in the present investigation, whether of seedlings or adult plants, the meristematic cells were conspicuously free from the fungus; this fact was also observed for Vaccinium by Freisleben (1934), and may be partly responsible for the delay in infection of seedlings in untreated soil. First the hypocotyl elongates and the cotyledons expand. Then there is a slight pause in development—the critical stage at which it has been claimed that uninfected seedlings are permanently arrested. Then root growth begins, and only after it has progressed far enough to leave a zone of mature vacuolated cells behind the meristem can infection take place. So far from infection being a prerequisite condition of root-formation, it is actually the other way about; the formation and growth of the roots are prerequisite conditions for infection.

THE EFFECTS OF SOIL AND SEED STERILIZATION

The endophytic fungus appears to be of universal occurrence in potting soil, but it seemed worth while to try the effect of its removal by initial sterilization of the soil. Accordingly, two small pots of peaty potting soil were autoclaved, and on September 17, 1935, one was sown with seeds of *Rhododendron decorum* which had been disinfected with calcium hypochlorite, while the other was sown with seeds which had been soaked in water only. On the following day a pot of unsterilized potting soil was sown with seeds of the same species disinfected with calcium hypochlorite. A preliminary trial had shown that, without sterilization of soil or seed, infection followed a similar course in *R. decorum* to that already described for *R. ambiguum*, and infected cells had been clearly seen some two months after sowing—in a pot sown on July 5, 1935, mycorrhiza-formation was found to have commenced in seedlings examined on September 12.

The three pots sown on September 17 and 18, as just described, afforded the three possible combinations of soil and seed sterilization, the object being to ascertain whether the occurrence of infection showed any correlation with the treatment of soil or seed. The pots were left in the seed-pit and the experiment continued for a period of seven months, the photograph (Pl. XXIII, Fig. 1) being taken on April 17, 1936, at which date examination yielded the following results:

Rhododendron decorum *in Peaty Soil. Result of Examination on April 17, 1936, Seven months after Sowing*

Soil.	Seed.	Root formation.	Endophytic infection.	Progress of seedlings.
sterilized	sterilized	+	—	good
,,	unsterilized	+	—	,,
unsterilized	sterilized	+	+	,,

In the unsterilized soil (centre, Pl. XXIII, Fig. 1) the roots had developed typical mycorrhiza, just as in normal pot culture. The seedlings had been infected from the soil, and the seed sterilization had made no difference.

In the other two pots moulds grew very freely at first, as always happens when autoclaved soil is exposed to the air; a number of seedlings were overgrown at an early stage, and died. This fact, together with sampling operations, accounts for the relatively smaller number of seedlings in these two pots than in the other, as shown in the photograph; such differences in numbers are not significant in the result of the experiment.

When the initial exuberant growth of moulds died down the seedlings in these two pots grew very similarly to those in the third, i.e. in untreated soil. Yet up to seven months from sowing, as recorded in the table, the roots were still free from endophytic infection, though by this time they were filling the pots and the seedlings had reached the stage of development shown in the photograph. Saprophytic hyphae were, of course, abundant in the soil, but it may be that the endophyte is not readily spread by air, being present in soil only as a vegetative mycelium; at any rate, it had not yet appeared and entered the roots.

In all pots the seedlings are recorded as having made good progress. The left-hand pot in the photograph has a much less dense mass of foliage than the other two, mainly because of the difference in numbers already mentioned, but comparison of the seedlings themselves gave no valid grounds for discrimination. There were much greater variations in size amongst the seedlings in any one pot than between comparable seedlings in different pots; in all three pots the leaves had a good green colour, and the seedlings appeared extremely vigorous. On the average, the seedlings in the central pot (untreated soil) were slightly taller than those in the other two; this is not surprising, as autoclaved soil, especially during the early phase of uncontrolled mould growth, is a much less favourable environment than untreated potting soil. The seedlings in the latter had established a lead before a state of relative biological equilibrium was re-established in the autoclaved soil. But this difference was small, and it was apparent that the seedlings in the autoclaved pots had not suffered any significant hindrance to their growth, as compared with those in the other pot.

The results of this pot-culture experiment are in agreement with those obtained by Christoph (1921) in a similar experiment with Calluna, which was criticized by Rayner (1922) in view of the incomplete control of sterility. It is recognized that this experiment has not the same value as those in which sterility is maintained (and which will also be reported), but taken in conjunction with them it does point strongly to two conclusions. Firstly, since the endophyte develops equally well in untreated soil whether the seed has been disinfected or not, and since it failed to appear—over a period of seven months —in sterilized soil, also irrespective of seed disinfection, it is apparent that infection proceeds from the soil, not from the seed. Secondly, the absence of the endophyte imposes no significant handicap on the growth of the seedlings.

PURE CULTURE ON AGAR MEDIA

The next step (though it had, in fact, been taken before that just reported) is to exclude soil infection by growing seedlings under aseptic conditions. Seeds were aseptically extracted, by the method already described, from the capsules of a considerable number of species, including *Rhododendron pholidotum*, *R. Nobleanum*, *R. desquamatum*, *R. ambiguum*, *R. decorum*, *R. ferrugineum*, and several unnamed garden hybrids. The non-appearance of hyphae in the early stages has already been recorded in the section dealing with shoot and seed infection. As the standard laboratory agar media did not seem to be very favourable to their continued growth, a number of seedlings were transferred singly to deep tubes of diluted (1·25 per cent.) malt agar. Pl. XXIII, Figs. 2–4, are successive dated photographs of one seedling of an unnamed hybrid. The seed was extracted and sown on oat agar on February 16, 1934, and the seedling transferred to 1·25 per cent. malt agar on March 19. Several others in this experiment were germinated on the more usual 2·5 per cent. malt agar, and transferred to the diluted medium on the same date; their subsequent development was similar to that of the example shown. Pl. XXIII, Fig. 2, taken on May 20, shows this seedling approximately three months after sowing and two months after its transference. As compared with soil culture, growth had been very slow; the seedling had reached the critical stage of development, and the curled cotyledons and practical absence of roots might suggest that it was incapable of further growth in pure culture. However, this seedling, and others of which it is typical, successfully passed that critical stage, and Pl. XXIII, Fig. 3, is a photograph of the same seedling some six weeks later. It had now formed roots and new shoot growth, and had entered on a more vigorous phase of development. On September 21, about six months after its transference to this tube, the same seedling had reached the condition shown in Pl. XXIII, Fig. 4, with a good green shoot and a well-developed, branched root-system. These roots were embedded in malt agar, a medium on which Ericaceous root fungi have been isolated (Freisleben, 1933–4), but during these six months of growth no hyphae had appeared in the medium, and when the seedling was subsequently removed and its roots stained and examined, no fungus was found in them; the same applies to a number of similar seedlings.

Since these plants were raised from seeds which had not been disinfected, but simply aseptically removed from the capsules, they confirm the opinion that the seeds are not infected within the parent ovary, and their subsequent development shows that Rhododendron can form roots in the absence of the endophyte, or of any other micro-organisms, just as Knudson claimed in the case of Calluna.

It has been stated that 2·5 per cent. malt agar is not very favourable to the growth of the seedlings, and this is illustrated in Pl. XXIII, Fig. 5. The photograph shows two tubes containing seedlings of *Rhododendron eritimum* grown from seed which had been disinfected with calcium hypochlorite; no micro-organisms appeared. These two tubes, and a number of others which yielded similar results, were sown on July 20, 1934, and the photograph was taken on December 19, when the seedlings were five months old. The tube on the left contained 2·5 per cent. malt agar, that on the right Knudson's Solution B agar, and the better development of roots on the latter is very obvious. In the malt agar culture small roots can be seen, particularly on the highest and lowest seedlings in the tube, but though root growth has not been entirely prevented it has evidently been greatly depressed. This result supports the idea that such inhibition of growth is due to unfavourable substrata, and at any rate not to the absence of the endophyte or of any other micro-organisms.

Again, Pl. XXIII, Fig. 6, shows root-formation by *Rhododendron decorum* on potato agar. The seeds were sterilized with calcium hypochlorite and planted on the agar slopes on September 12, 1935; the photograph was taken on

December 16, so that the seedlings were three months old. They were still at an early stage in the rather slow development usual in such cultures, and only one root had so far appeared, but it shows the healthy, glistening white appearance of roots produced in pure culture on suitable media.

PURE CULTURE ON OTHER MEDIA

Reference has been made in the section on media to the use of sterile sand irrigated with Knudson's Solution B. Aseptically extracted seeds of *Rhododendron ambiguum* were sown on this medium on December 6, 1935, and the flask left in an unheated greenhouse. Pl. XXIII, Fig. 7, is a photograph of four of these seedlings raised on August 6, 1936, eight months after sowing. They seem surprisingly small, but this is in part explained by the fact that the seeds lay dormant all winter; with rising temperature in early summer they germinated, and the photograph really shows the result of a few months growth in the summer of 1936. At this stage the seedlings were small but healthy, with leaves of a good green colour. They had developed roots in which careful examination revealed no fungus. The sterility of the medium was simultaneously verified by transferring samples to 2·5 per cent. malt agar, on which no growth occurred.

These seedlings, being grown from aseptically extracted seeds, without sterilization, confirm the opinion that infection is not seed-borne, as well as the ability of the seedlings to form roots and establish themselves in pure culture.

A similar experiment was carried out with *Rhododendron decorum*, except that the seeds in this case were obtained from a seed-packet, and were therefore disinfected with calcium hypochlorite and sown, in the first instance, on 2·5 per cent. malt agar. This was done on November 27, 1935, and the seeds were left for a week on the agar plate at room temperature to confirm the absence of any contaminants. On December 5 the seeds, which had remained quite clean, were transferred to a flask of sand and Knudson's Solution B, which was left in the unheated greenhouse till August 6, 1936, when the four seedlings shown in Pl. XXIII, Fig. 8, were raised from it for examination. These seedlings were larger than those of *R. ambiguum* raised from sand at the same time. This is in part a specific difference, *R. decorum* being a more free-growing species than *R. ambiguum*; in addition the seeds of *R. decorum* did not remain dormant throughout the winter, though their growth during this period was slow. Their earlier germination may have been due to the accelerating effect of calcium hypochlorite.

Here again no fungus could be found in the roots, nor in the sand by transferring samples to malt agar. Since the seed had been disinfected this experiment has no bearing on the question of seed infection within the ovary, but it confirms again the ability of fungus-free seedlings to establish themselves and form roots.

Rhododendron decorum was also used for an experiment with Molliard's cotton-wool medium. The seeds, from a seed-packet, were disinfected with calcium hypochlorite and sown directly on the cotton-wool on January 10, 1936. Pl. XXIII, Fig. 9, is a photograph of this tube taken on June 17, some five months after sowing, during which time the tube, and similar cultures of *R. eritimum*, *R. ferrugineum*, and *R. Davidsonianum*, had been kept at room temperature. Access of light was provided by keeping the tubes near a window, but their position was chosen and varied to prevent much direct sunlight reaching them. Under these conditions the seedlings had formed vigorous shoots of a good green colour, while their roots had freely penetrated the cotton-wool. The well-developed roots of one seedling lay mainly between the cotton and the wall of the tube, and could therefore be photographed, as seen in Pl. XXIII, Fig. 10, which was taken on August 11, 1936. Two days later (Aug. 13) and thus some seven months after sowing, sample seedlings were removed and their roots, including those seen in Pl. XXIII, Fig. 10, were stained and examined. No infection could be seen. Samples of the cotton-wool were transferred to malt agar and remained sterile.

This, then, is a further example of the successful growth of pure-culture seedlings.

When previous workers have recorded similar results, mainly for Calluna and Vaccinium, it has been suggested by Rayner (1922, 1925, 1929*b*) that under artificial cultural conditions the fungus is present in an extremely attenuated condition, permitting the development of the seedlings, which could not succeed in its total absence, but not forming typical mycorrhiza, and that it has been overlooked. It is extremely unlikely that this can explain all the negative results that have been recorded, but in any case there is now available a controllable medium in which it is known that typical mycorrhiza does form. This is the peat and sand mixture already referred to in the section on media. Freisleben (1933, 1934, 1935) used this medium for experimental synthesis of mycorrhiza, by transplanting to it pure-culture seedlings of various Ericaceae, including Rhododendron species, and simultaneously inoculating the substratum with the endophytic fungi which he had isolated from Vaccinium roots. He records the fact, borne out by photographs, that infection by the endophytes in this medium took the form of a copious mycorrhiza-formation, indistinguishable from that occurring in nature. It may therefore be maintained that negative results obtained with this medium are not subject to the criticism that the fungus might be so attenuated as to escape the most careful search.

It may be recorded at once that the results obtained in the present investigation by the use of this peat and sand medium have been in complete agreement with those already discussed. Seedlings can, and do, form roots and grow successfully in the absence of the endophyte, verified by careful examination of the roots, and in the absence of any other fungi, verified by transferring samples of the substratum to malt agar. This is of interest, because Freisleben, using this medium, was disposed to agree with Rayner as to the failure of pure-culture seedlings to form roots, though he found that this condition was relieved by any non-parasitic fungi, and not only by Ericaceous root-fungi. In the present investigation roots have been formed quite successfully when no fungi were present at all.

It is quite true that under some conditions fungi appear to be beneficial to the seedlings, even though they are not indispensable, and in this medium they certainly do no harm. The largest seedling produced in any of these cultures was one of *Rhododendron decorum* which was grown alone in a 250 c.c. flask, in which the peat-sand substratum had been contaminated with Penicillium. The seed was sterilized with calcium hypochlorite and planted on potato agar on September 12, 1935. On January 17, 1936, four months after sowing, this particular seedling was transferred to the flask culture, which became contaminated, as occasionally happened while introducing seedlings, with the ubiquitous Penicillium. Such flasks were not discarded, but kept for observation of any effect which the fungus might produce on the growth of the seedlings, Penicillium being one of the fungi recorded by Freisleben (1934) as relieving the inhibition of growth which he found in pure-culture seedlings.

At the time of its transference this seedling had but one short lateral root, and no visible epicotylar growth. Pl. XXIII, Fig. 11, is a photograph of the same seedling, still in its flask, some seven months later, namely, on August 5, 1936. On August 11 it was removed from the flask and found to have an extremely well-developed root-system (Pl. XXIII, Fig. 12); the leaves were of a fine dark green. Penicillium was known to be present, and was recovered in apparently pure culture on transference of samples of the medium to malt agar. On microscopic examination of roots the hyphae and spores of the saprophyte, were found on the surface, but the root-cells were clean and empty, without any sign of mycorrhiza-formation.

This seedling, then, had been conspicuously successful in the absence of the endophyte, but in the presence of Penicillium.

But in this medium it does not appear that seedlings grown in the presence of fungi have any real advantage over pure-culture seedlings. On January 17, 1936, when the seedling described above was transferred from potato agar, two other cultures were set up in which seeds of *Rhododendron decorum* were sown directly on peat-sand mixture in two 250 c.c. flasks. In one were sown about a dozen seeds which had been soaked in water, sterilized by immersion in calcium hypochlorite for about forty minutes, and transferred directly without rinsing; in the other were sown a similar number of seeds which had been soaked in water, and then rinsed in several changes of sterile water, without sterilization. These seeds were taken from a seed-packet, and the washing of the second set proved insufficient to prevent contamination. Penicillium developed in the substratum, and was recovered in apparently pure culture when the flasks were opened on June 24, 1936, i.e. five months after sowing. It is, however, possible that other saprophytic organisms were present, but were masked by the Penicillium.

On the same date (June 24) sample seedlings were raised and photographed (Pl. XXIII, Fig. 13). The central seedling is an average representative of the flask containing Penicillium; the other two are a large and a small seedling from the pure-culture flask, in which the average size did not differ appreciably from that in the contaminated flask. After photographing, these seedlings were stained and carefully examined. The roots of that from the infected flask bore hyphae superficially, but not internally; no fungus could be found on or in the roots from the other flask, nor was any revealed by transferring samples of the substrate to malt agar.

Now, the seeds sown in the infected flask had not been sterilized, so that mycorrhiza should have developed if the endophyte were seed-borne. Also comparison of the seedlings shown in Pl. XXIII, Fig. 13, and the recorded similarity in the average development of these two sets of seedlings of equal age, show that in this medium the presence of fungi of any kind is not only unnecessary; it does not even confer any apparent advantage. And this experiment was carried out with a medium in which the presence of the endophyte results in typical mycorrhiza-formation, and is therefore not liable to be overlooked.

Conclusions

The three controversial questions in relation to Ericaceous mycotrophy mentioned at the beginning can now be answered in the case of Rhododendron:

1. The endophyte is confined to the roots, and does not penetrate the subaerial parts of the plant. This has been determined mainly by microscopic examination, supported by the failure of attempted isolations, and by the absence of seed infection (see below).

2. As implied by 1, the endophyte is not seed-borne. This has been shown by the fact that the endophyte did not appear in any of the cultures from aseptically extracted seed, nor in any culture where the substratum itself had been sterilized whilst the seed had not, and this remained true even when the substratum was one in which the endophyte is known to form typical mycorrhiza if present.

3. Infection by the endophyte is not an obligate condition of development of the higher plant, which in fact can form roots and establish itself in the total absence of any micro-organisms. This is demonstrated by the success recorded for pure-culture seedlings on a considerable variety of substrata.

Discussion

What, then, is the significance of the mycotrophic habit in these and related plants? In Rhododendron, if the above conclusions are sound, it is plainly not of the vital character envisaged by Rayner for the Ericaceae in general, and for Calluna in particular.

A recent paper by Burges (1936) supports the belief that mycorrhizal fungi are essentially parasites, and that no mutualistic relation is involved. In the same paper it is suggested that soil fungi generally, including perhaps the mycorrhizal fungi, break down organic matter to a level at which some of it is water-soluble, and can be absorbed directly by the roots of some of the higher plants.

The conclusion that mycorrhizal fungi are essentially parasites has been reached by other workers in this field, and it is the one which seems to accord with the results now presented. This is not inconsistent with the accepted fact that the presence of fungi is sometimes beneficial to the higher plant. It is known that plants do not generally grow well in completely sterilized soil, and that applies to all the higher plants, not only to those which form mycorrhiza. The metabolism of micro-organisms plays an important part in soil fertility. If this role, in the case of soil fungi, consists of breaking down organic matter to a level at which some of it can be absorbed, in accordance with the suggestion just quoted, then it could be understood how, on some substrata, if the available nutriment is scanty, the presence of an endophytic or other fungus might permit the development of the green plant by making available food materials which could not otherwise be used, and this might apply to some of the substrata which have been used in mycorrhiza research, especially agar media. On the other hand, if the substratum already contains adequate nutriment in an available form, as where soil water or a suitable solution of salts has been incorporated in the medium, then it would be expected that the presence of a fungus would be quite unnecessary, and would even confer no advantage, as found in the present investigation.

Poor growth in some media must be due to a positively inimical factor, and not merely to lack of available food material; this applies, for example, to 2·5 per cent. malt agar, where the inhibitory effect was considerably relieved by dilution of the medium, but the availability of nutriment may be the key to understanding of the otherwise anomalous records (especially by Rayner, 1915, and other papers) of improved growth on one and the same medium on the introduction of a fungus.

It appears, then, that the root fungi have no specific importance to the higher plant. The feature which distinguishes them from other soil fungi is simply a certain mild degree of parasitic activity towards these plants, whereby they are able to invade mature root-cells and, though held in check by the host plant from further spread or damage, to survive as parasites within those cells, where they produce the coiled masses of hyphae which are the most characteristic feature of this type of mycorrhiza.

SUMMARY

In nature the roots of Rhododendron species regularly contain an endophytic fungus; the mycorrhiza is of the same type as in Calluna, Vaccinium, and the majority of the Ericaceae.

There are three questions in particular in the mycotrophy of the Ericaceae on which opinion has been divided. These questions are investigated by

microscopic and cultural methods, and evidence is presented to justify the following answers in the case of Rhododendron: (1) The endophyte is confined to the roots, and does not penetrate the sub-aerial parts of the plant. (2) As implied by 1, the endophyte is not seed-borne. (3) Infection by the endophyte is not an obligate condition of development of the higher plant, which in fact can form roots and establish itself in the total absence of any micro-organisms.

The significance of the mycotrophic habit is briefly considered in the light of these results, which lead the writer to support the view that the endophyte is a relatively feeble parasite, and is of no specific importance to the higher plant.

This investigation was carried out in the Mycology Department of the University of Edinburgh. The writer is particularly indebted to Dr. Malcolm Wilson for constant help and criticism, as well as for the original suggestion that the extensive Rhododendron collection in the Royal Botanic Garden, Edinburgh afforded a good opportunity to study this problem. The writer would also thank Sir William Wright Smith, Regius Keeper of the Garden, and Mr. R. E. Cooper, Curator, for access to plant material.

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EXPLANATION OF PLATE XXIII

Illustrating Dr. Gordon's paper on 'Mycorrhiza in Rhododendron'

Fig. 1. *Rhododendron decorum*, 7 months old. Left: sterilized seed; sterilized soil. Centre: sterilized seed; unsterilized soil. Right: unsterilized seed; sterilized soil.

Fig. 2. *Rhododendron* hybrid No. 1, growing in 1·25 per cent. malt agar: sown, February 16, 1934; transferred to tube shown, March 19, 1934; photograph taken, May 20, 1934.

Fig. 3. The same seedling: July 3, 1934.

Fig. 4. The same seedling: September 21, 1934.

Fig. 5. *R. eritimum*, 5 months old. Left: on 2·5 per cent. malt agar. Right: on Knudson's Solution B agar.

Fig. 6. *R. decorum*, 3 months old: root-formation on potato agar (r = root).

Fig. 7. *R. ambiguum*, 8 months after sowing: seedlings rooted in aseptic sand culture with Knudson's Solution B.

Fig. 8. *R. decorum*, 8 months after sowing: seedlings rooted in aseptic sand culture with Knudson's Solution B.

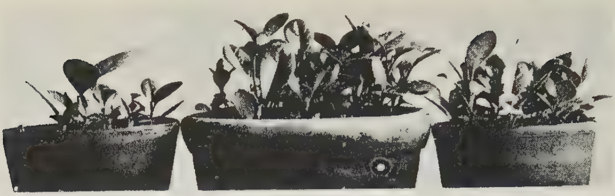
Fig. 9. *R. decorum*, 5 months old: aseptic culture on Molliard's cotton-wool medium.

Fig. 10. Closer view of same tube 2 months later, showing roots.

Fig. 11. *R. decorum*, 11 months old, in peat and sand infected with Penicillium.

Fig. 12. Same seedling removed from flask to show root-system.

Fig. 13. *R. decorum*, 5 months old, grown in peat and sand. Centre: from culture infected with Penicillium. Left and right: from aseptic culture.



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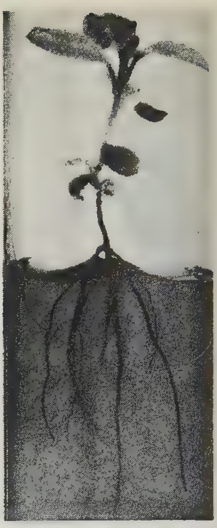

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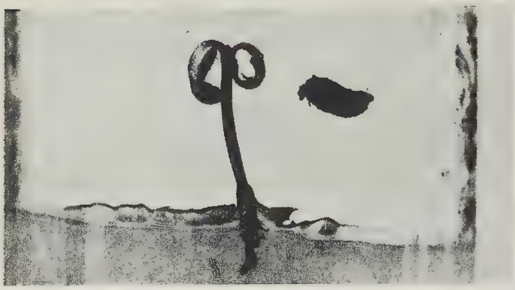

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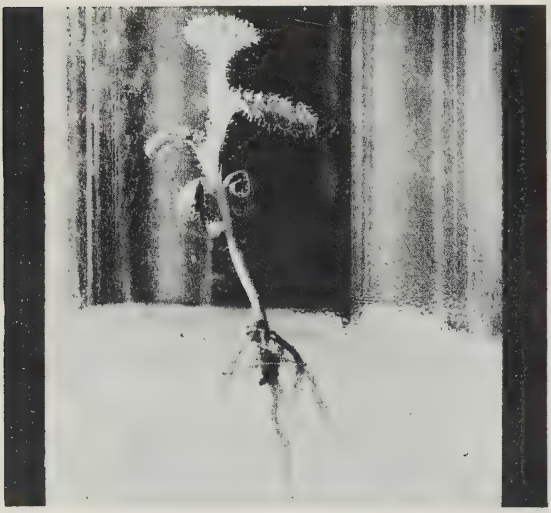

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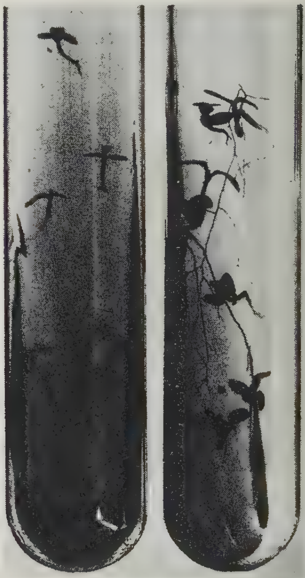

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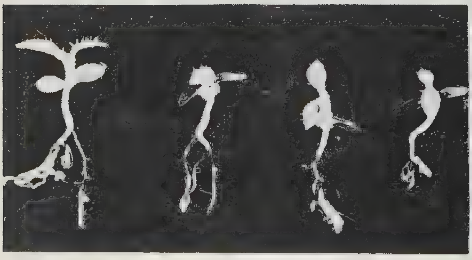

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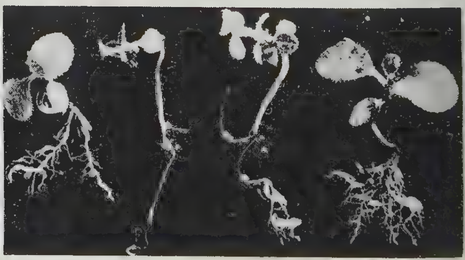

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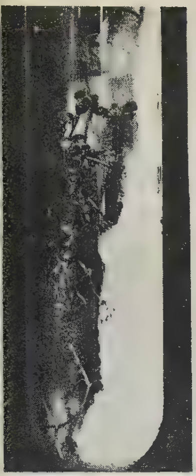
GORDON — MYCORRHIZA IN RHODODENDRON.



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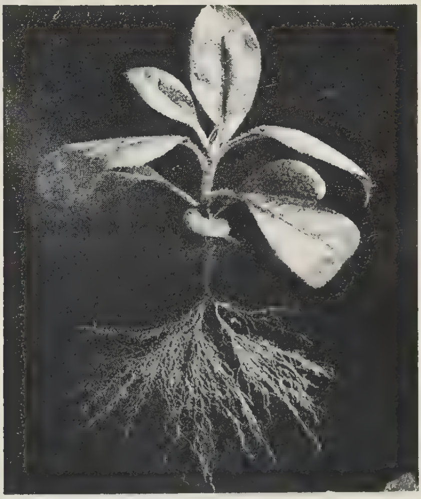

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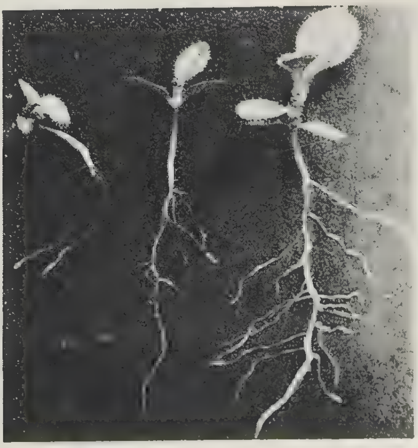

13

Huth, Stubbs & Kent.

Experiments on the Influence of Parathyroid and Thyroid Hormones upon the Growth of Seedlings

BY

E. D. BRAIN

RECENT workers on the influence of animal hormones and plant growth have tested a number of substances. Hávás and Caldwell (1935) have reviewed the previous work so that repetition here is unnecessary, but it may be mentioned that they omitted reference to the work of Davis (1934) on the effect of thyroxin on bulbs and seedlings. She found that thyroxin hastened flowering in Narcissus and Allium and retarded root growth in Pisum seedlings. No reference to previous work on parathyroid hormone and plant growth has been found. It therefore seems worth recording the results which the writer has found when seedlings have been grown in dilute solutions of parathyroid and thyroid hormones.

METHODS

The hormone preparations used were Paine and Byrne's Ltd. 'Pabryn' brand. The parathyroid solution contained twenty Collip units per cubic centimetre. A Collip unit is the standard unit for parathyroid extract as defined by Collip, the pioneer worker on the parathyroid hormone (see Harrow and Sherwin, p. 26 (1934)). The thyroid solution was standard solution prepared from fresh gland. The seeds were germinated and then placed with the roots dipping into water to which small quantities of the hormone solutions were added. Measurements were taken with a millimetre rule. Experiments were not performed at constant temperature, but a record of maximum and minimum temperatures was kept, and since all series of treated plants and controls which were compared had similar conditions, it was not considered that slight variations of temperature were harmful.

PARATHYROID HORMONE

Seeds of *Avena sativa* (Sutton's Winter White Oats) were germinated, and when the roots of the seedlings were about 1 cm. long they were placed on perforated zinc with the roots dipping into solutions of parathyroid hormone in distilled water. Controls were grown in distilled water. Several series of seedlings in different dilutions of parathyroid in tap-water and distilled water showed the same results, i.e. a hindrance to the growth in length of roots in the parathyroid solutions. The roots of treated seedlings sometimes curved at the tips and grew along parallel to the surface of the solution, and they seemed thicker than the controls. No difference was noted in the number of roots produced on treated and untreated seedlings. Cholodny (1931) has found increased thickness in maize roots after sticking tips of coleoptiles on the root-tips. He gives striking photographs on page 210 in his paper and attributes the increased size in the growing region of the cortex to the auxin absorbed from the coleoptile tips. Kögl, Haagen-Smit, and Erxleben (1934) have shown that auxin-*a* solutions have a marked hindrance effect on growth in lengths of roots of Avena seedlings when they are grown in them. It is interesting to find that a specifically animal hormone such as parathyroid should have a similar effect on plant growth. Auxin-*a* is found in, and may be isolated from, animal excretions but its ultimate source is the plant taken as food (Kögl, 1933).

TABLE I

Results for Parathyroid Hormone Experiments with Avena sativa *Seedlings*

			Average length in cm.		
Solution used.	Time in days.	Number of plants.	Longest root.	Coleoptile.	1st leaf.
0·4 c.c. parathyroid in 100 c.c. distilled water	6	9	5·2	1·8	2·2
Control	6	11	6·0	1·5	2·2
Temperature 21–15° C. Percentage difference:			−13·3	+20·0	0
0·1 c.c. parathyroid in 100 c.c. distilled water	6	9	4·6	1·1	1·0
Control	6	11	6·0	1·5	2·2
Temperature 21–15° C. Percentage difference:			−23·3	−26·6	−54·5
0·3 c.c. parathyroid in 250 c.c. tap-water	6	5	10·9	1·9	6·7
Control	6	5	13·3	2·3	10·5
Temperature 22–20° C. Percentage difference:			−18·0	−17·3	−36·1
0·15 c.c. parathyroid in 250 c.c. tap-water	9	12	11·3	3·8	14·3
Control	9	10	12·8	4·0	14·1
Temperature 22–20° C. Percentage difference:			−11·7	−5·0	+1·4
0·3 c.c. parathyroid in 250 c.c. distilled water	9	11	3·7	3·5	10·5
Control	9	11	4·3	3·2	10·0
Temperature 22–20° C. Percentage difference:			−14·0	+9·4	+5·0

+ and − signs before percentages denote differences of treated plants above or below control plants.

The results of these experiments are summarized in Table I. It will be noted that in each experiment the average length of roots is consistently shorter for the treated plants than the controls, whereas the average lengths for coleoptile and leaf are either below or above that of the control plants. The results are expressed as percentage differences between the two averages. The + or − signs indicate whether the average of the treated plants is greater or less than controls.

THYROID HORMONE

Seeds of *Phaseolus multiflorus* (Carter's Streamline Runner Bean) were germinated in damp moss and when the roots measured 2·0–4·0 cm. long the seedlings were transferred to jars of distilled water. Twenty-four hours later 0·1 c.c. thyroid solution was added for every 100 c.c. distilled water. Controls were kept in distilled water. Daily measurements of root and shoot growth were taken. Plants in thyroid solution showed less rapid growth in the roots than control plants. The hindrance effect was noticeable five hours after the thyroid solution was added. The roots of treated plants often curled at the tips and the main roots were shorter and thicker than the controls; the side roots of treated plants did not grow so rapidly and began branching earlier than those of the controls. Epicotyls, in some cases, seemed stunted and the side shoots in the axils of the cotyledons developed, in other cases they grew normally in the thyroid solution. When the experiments were prolonged, after three or four days the thyroid solutions became turbid and the roots coated with slime. After the solutions were changed and the plants transferred to clean jars, the same infection developed. In spite of precautions for sterilization and washing the seedlings in mercuric perchloride solution the thyroid solutions became turbid after several days, whereas controls remained perfectly clear. This development of infection is probably due to the organic nature of the thyroid extract and has been referred to by other workers with hormone preparations. Davis (1934) found similar conditions when growing Pisum seedlings in Pfeffer's solution to which thyroxin was added, and Tincker (1935) found that bacterial slimes developed when experimenting with ketohydroxyoestrin. Imshenetskii (1936) has found that thyroid extract stimulates spore formation and increases reproduction in yeasts and moulds. This contamination of the solutions makes it necessary to discount later readings, but the marked decrease in the first few hours after treating plants with thyroid solution indicates an inhibitory effect of the hormone on root growth. In all series of experiments this effect was evident. Table II gives examples of a series of readings taken and Table III the averages of measurements for a number of experiments.

Three plants grown in thyroid solution with short roots were transferred to distilled water and made a rapid recovery, the roots and side roots growing on.

These results for Phaseolus correspond with Davis's (1934) work on thyroxin, which she finds retards growth of roots in Pisum.

The retardation in growth of roots made it seem advisable to attempt some means of testing the effect of thyroid on the shoot alone, since the impaired root growth might of itself affect the shoot. For this purpose bean plants were grown in damp moss and the shoots cut off at the external collet and placed in distilled water. After twenty-four hours increase in length was measured and half the shoots were transferred to solutions containing 0·1 c.c. thyroid solution in 100 c.c. distilled water. Solutions and water were renewed each day. There was little difference as Table IV indicates.

TABLE II

Results of Thyroid Hormone Experiments. Phaseolus multiflorus *Seedlings*

Radicle.	Control.				Treated.			
Initial length (cm.)	3·5	3·0	2·6		4·0	4·0	4·0	3·5
Increase in length, cm. in 24 hours	2·4	2·0	1·0	In distilled water	1·0	2·5	2·0	1·6
„ 5 „	0·6	1·3	0·6	+ thyroid	0·0	1·5	0·5	0·4
„ 24 „	3·0	3·2	2·0	„	0·5	0·8	0·8	1·2
„ 24 „	2·0	1·0	2·0	„	1·2	1·2	0·4	0·5

Epicotyl.	Control.				Treated.			
Increase in length, cm.: in 24 hours	1·5	—	—	+ thyroid	—	—	1·5	1·2
„ 24 „	1·2	1·5	3·1	„	1·3	—	3·0	3·3
„ 24 „	1·8	2·9	1·3	„	1·9	0·4	3·0	3·0
„ 24 „	2·8	3·1	6·2	„	0·2	0·8	3·5	3·5

Temperature 23–18° C.

TABLE III

Results of Thyroid Hormone Experiments. Phaseolus multiflorus *Radicles*

	Average growth (cm.) Control (11 plants).		Treated (15 plants).	Percentage difference.
1st day	2·0	In distilled water	1·78	−11·0
2nd day	2·25	+ thyroid	0·91	−59·5
3rd day	2·25	„	0·86	−61·7

A minus sign indicates a percentage below the controls.

TABLE IV

Experiments with Cut Shoots of Phaseolus multiflorus *in Thyroid Hormone Solution*

Increase in length (cm.)

	Control.				Treated.		
Initial length (cm.):	19·0	16·4	10·5		14·6	9·9	6·6
1st day	1·0	1·6	2·4	In distilled water	1·4	1·6	1·4
2nd „	3·0	3·2	3·3	+ thyroid	1·7	3·4	2·0
3rd „	7·2	3·4	3·3	„	2·0	3·3	1·8
4th „	6·1	1·9	1·0	„	2·3	2·5	1·3
Percentage increase	91·0	61·5	95·0	„	57·5	109·0	98·4

Temperature 22–19° C.

Avena sativa.

Some experiments were performed with *Avena sativa* (Sutton's Winter White Oats). Several series were grown in dilute Knop's solution and reduction in growth of roots, coleoptiles, and first leaves was evident in plants in solutions containing 0·3 c.c. thyroid hormone in 225 c.c. These results are not regarded as reliable as the solutions were infected. Two more series using tap-water showed contradictory results. A series of seedlings treated with 0·3 c.c. thyroid hormone per 100 c.c. showed 33 per cent. less growth in roots, and the other series treated with 0·1 c.c. thyroid hormone per 100 c.c. showed 100 per cent. more growth in roots than the controls for the day following the addition of the thyroid solution.

Radish (Carter's Mixed Turnip).

Seeds were sown in pots of sand and each pot given an equal quantity of water. On germination of the seeds, some of the pots were watered with Knop's solution to which 0·05 c.c. thyroid solution was added per 50 c.c., others were given Knop's solution only. This was repeated three days consecutively. At the next application, after two days' interval, the thyroid dose was increased to 0·1 c.c. per 50 c.c. Knop's solution. Three days later ten seedlings treated with thyroid and ten control seedlings were transplanted to boxes of soil. The others were repotted in fresh sand. The treatment with thyroid was continued, the plants in the boxes being given separate doses of 0·1 c.c. thyroid solution in 10 c.c. water, the soil being removed from above the roots before each application.

TABLE V

Experiments with Thyroid Hormone. Radish Seedlings

	Average length (cm.) Control (15 plants).	Treated (20 plants).	Percentage difference.
	Grown in sand.		
Root	6·1	7·6	+24·5
Hypocotyl	4·2	4·2	0·0
Leaf	6·2	6·7	+8·0
	(10 plants).	(10 plants).	
	Grown in soil.		
Root	7·3	6·0	−17·8
Hypocotyl	5·4	4·4	−18·5
Leaf	11·6	10·6	−8·6

Owing to unfavourable weather conditions the radishes did not mature at all well. After five weeks a comparison of those grown in the sand showed little difference in size of roots or stem in treated and untreated plants. Roots and leaves of treated plants were slightly longer than controls.

Measurements of the plants which had been transplanted to soil, after two weeks, showed that the thyroid-treated plants were slightly smaller than the controls (see Table V). The results are not regarded as conclusive owing to unsatisfactory conditions for the experiment, but it may be noted that the treated plants which show less growth were receiving bigger dosage with thyroid than the series grown in sand. Although the results for *Avena sativa* and radish seedlings need confirming by further experiment, it is felt that they indicate the fact that there may be different effects produced in different species by the same hormone substance; and, that different concentrations of dosage may yield opposite effects in the same plant.

DISCUSSION

Hávás and Caldwell (1935) state that the action of hormones seems to vary with the concentration of hormone itself and may produce different effects in the same plant according to whether the concentration is high or low. This consideration complicates the results and emphasizes the difficulty which is felt in forming definite conclusions on the effects of the hormones, and may explain the contradictory results which the various workers have found. However, there seems to be agreement in certain results for thyroid hormone. Davis (1934) recorded earlier flowering in Narcissus and Allium after treatment with thyroxin, and similar effects in root growth in Pisum seedlings to the results described above for Phaseolus. Niethammer (1927) found a stimulatory effect of thyroid extract on opening buds of various dormant shoots and on germinating seedlings.

Budington (1925) found greatly decreased growth in roots of Narcissus and Allium grown in nutrient solution containing thyroid extract. In order to find if thyroid gland extract could cause precocious development of unspecialized plant tissue, he examined the development of the root-cap in roots grown in nutrient media containing thyroid extract. His measurements showed that differentiation of special root-tissues extended nearer the tip in roots grown in solutions containing thyroid extract, than in controls. He assumed that the freshly growing tissues of the root were comparable to those in larval amphibia, which would respond to dosage with thyroid extract by precocious development of tissues, and suggested that thyroid caused precocious development in both. He also stated that hastened specialization of tissues in amphibian larvae, dipterian larvae, and protozoa were all accompanied by undersize, and pointed out the correlation of decreased root length and precocious development of the root-cap, which he demonstrated in Narcissus. The experiments recorded above show marked decrease in length of root in beans treated with thyroid extract and in *Avena sativa* treated with parathyroid extract, but no examination of internal morphology has been attempted. If a comparison of plants and amphibia is legitimate it is interesting to note that Thompson and Huxley record retarded growth in axolotls resulting from treatment with parathyroid hormone. Such comparisons indicate a similarity in the response of plant and animal protoplasm to certain hormones.

SUMMARY OF RESULTS

In the experiments described seedlings were grown in solutions of parathyroid and thyroid hormone. Decrease in length of roots was marked in *Avena sativa* in parathyroid solution and in *Phaseolus multiflorus* in thyroid solution. Coleoptiles and leaves of *Avena sativa* do not seem to be affected by parathyroid solution.

Epicotyls of beans were stunted in some cases when plants were grown in thyroid solution, but experiments with cut shoots did not indicate any effect of thyroid solution on the growth of the shoot.

Experiments with radishes and *Avena sativa* seedlings treated with thyroid solution did not give conclusive results, but indicate that a dilute solution may stimulate growth though a stronger one may retard it.

Miss M. Williams, M.Sc., F.L.S., has very kindly given me some help in the preparation of this manuscript, and I am indebted to Dr. T. L. Prankerd, F.L.S., also, for her kindness in discussing the work.

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The Interaction of Factors in the Growth of Lemna

XI. The Interaction of Nitrogen and Light Intensity in Relation to Growth and Assimilation

BY

H. L. WHITE, Ph.D.

(*From the Research Institute of Plant Physiology, Imperial College of Science and Technology, London*)

With twelve Figures in the Text

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INTRODUCTION

Experimental procedure. The results of preliminary investigations of the effect of potassium, nitrogen, and light intensity on the growth of Lemna colonies under rigidly controlled conditions (White, 1936, 1936*a*, 1936*b*) opened the way to the further study of the interaction of these factors. The present communication concerns the interaction of nitrogen and light intensity.

Quantitatively similar results were not obtained in previous experiments carried out at different periods of the year (White, 1936*b*) and all treatments compared in this investigation were therefore carried through simultaneously. The apparatus of Ashby (1929) for the continuous circulation of nutrient solution at constant temperature through four dishes, each under a different intensity of light, was modified. In the present experiment the plant colonies were grown in 30 c.c. Pyrex beakers, four beakers being placed on the floor of each dish within the field of a single lamp. Each dish was loosely covered by a glass plate to prevent dust contamination. The dishes were filled to a level just below that of the rims of the beakers with distilled water, which

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was continuously aerated and circulated for temperature equalization and to preclude the possibility of carbon dioxide limitation. The nutrient solution (pure inorganic salts and glass-distilled water)[1] was changed throughout the experiment at intervals of twelve hours. Duplicate sets of beakers stood in the dishes beside the experimental sets to ensure that the colonies were transferred to fresh solution without any change in temperature. Fluctuation from a constant temperature of 25° C. was limited to half a degree by a mercury thermo-regulator, with a platinum contact in an atmosphere of hydrogen, which actuated carbon-filament lamps below a water-bath. Continuous illumination was supplied from gas-filled lamps of different wattage, jacketed in round-bottomed beakers containing flowing cold water. The intensity of light at the surface of the colonies was estimated by a Holophane lumeter and the positions of the beakers under each lamp alternated to cancel irregularities in the light-field. The nitrogen supply was varied by altering the concentration of a mixture of calcium and magnesium nitrates.

The effect on growth of the sixteen possible combinations of four nitrate concentrations with four light intensities was investigated simultaneously. The levels of the factors[2] studied were as follows:

nitrate concentration: 4·0, 1·0, 0·5, and 0·25 mg. nitrogen per litre.
light intensity: 300, 200, 100, and 50 ft.-candles.

Growth measures. Area was estimated by drawing an enlarged outline of a sample focused on a ground glass screen and tracing with a planimeter as described by Ashby, Bolas, and Henderson (1928), and dry weight by drying *in vacuo* under standard conditions and weighing on a microchemical balance as described by Su and Ashby (1929). The capacity of the sampling apparatus sufficed for the estimation of area and dry weight from half the number of treatments at a single sampling. When calculating the total number of fronds composing the colony, allowance is made for the withdrawal of samples for area and weight determinations and for the random removal of plants to prevent overcrowding. The relative rates of increase of the colonies (percentage increase per day of twenty-four hours' continuous illumination) are estimated from differences between natural logarithms of frond number.

The desirability of replication of the treatments for the purpose of statistical analysis of the results was realized, but it would have required the design and construction of more extensive apparatus than was available and would have placed the work beyond the scope of a single investigator. Even with a single colony to each treatment the work of counting, sampling, preparing fresh solution, &c., necessitated the attention of the investigator for fourteen hours daily and was only made possible by strict adherence to a time-table. In order to enable an estimate of error to be made each sample consisted of eight plants of two fronds each, taken from random positions in the colony. From variation within colonies the significance of differences between colonies may be assessed. In the absence of replicate colonies it does not necessarily follow that the differences found are due to the treatments applied, though in a constant environment and with colonies derived from the splitting of a single clone the probability that this is so must be high. The use of variance within colonies instead of variance between colonies in order to test the significance of effect of treatment is considered in the section on frond area.

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[1] $CaH_4(PO_4)_2$ 0·101 gm.: $MgSO_4$ 0·255 gm.: K_2SO_4 0·334 gm.: Fe_2Cl_6 0·002 gm.: $Ca(NO_3)_2$ $4H_2O$ 0·232 gm.: $Mg(NO_3)_2$ $6H_2O$ 0·153 gm.: water 1,000 c.c.

[2] The interaction of higher light intensities and nitrogen supply will be considered in a later communication.

The plants used for the experiment, which lasted for sixteen days, were obtained by the division of a colony grown for three weeks previously at 25° C. under continuous illumination of 300 ft.-candles. Rates of increase in frond number characteristic of treatment were established early in the experiment, but relatively long periods were required to bring out the effect of treatment on frond area and dry weight. During the first eight days only slight differences were noted, which do not appear to exceed the range of random error. The mean values recorded in Tables II, III, and VIII refer, therefore, to the second half of the experiment only.

II. Experimental Results

Rate of increase in frond number. After the first two days the rate of increase in frond number became *exponential* in all colonies and remained so for the major part of the experiment. At the end of the experiment there were signs of a falling off with time in those colonies with severe nitrogen starvation.

Table I

Relative Rate of Daily Increase of Frond Number for all Combinations of Four Nitrate Concentrations and Four Light Intensities

Light intensity.	Nitrogen (mg. per litre).			
	0·25.	0·5.	1·0.	4·0.
300 ft.-candles	0·145	0·160	0·180	0·199
200 „	0·134	0·162	0·173	0·171
100 „	0·119	0·122	0·122	0·126
50 „	0·104	0·092	0·085	0·103

The mean indices of relative rate of increase in frond number per day are given in Table I. The maximal rate of increase in frond number is obtained with the combination of maximal light intensity and maximal nitrogen supply, reduction in the value of either factor being associated with a falling off in multiplication rate. The interaction of the factors may be studied in Fig. 1, showing the differential effect of nitrogen supply for four levels of light intensity, and Fig. 2, showing the differential effect of light intensity for four levels of nitrogen supply. The effect of increasing nitrogen supply at the maximal level of 300 ft.-candles is represented in Fig. 1 by a smooth curve, apparently logarithmic in type. At 200 ft.-candles the influence of nitrogen supply is less marked, and at 100 ft.-candles variation in the nitrogen factor has no effect on growth. With further reduction in light intensity to 50 ft.-candles increasing nitrogen supply appears to have a detrimental effect, for increase in nitrogen concentration from 0·25 to 1·0 mg. per litre is associated with a fall in multiplication rate.

The effect of increasing light intensity in Fig. 2 is represented by curves of similar type for all levels of nitrogen supply. The dispersion of the points is greater at 50 ft.-candles than at 100 ft.-candles, suggesting that the curves intersect about the latter value and that at lower light intensities maximal growth rate is associated with minimal nitrogen supply. The experimental value at 50 ft.-candles and 4·0 mg. nitrogen per litre is out of position and examination of both diagrams suggests that the colony with this combination of treatments should have been represented by a value of about 0·085. The trend of the curves of Fig. 1 suggests that reduction in nitrogen supply below 0·25 mg. per litre would result in intersection of the light intensity curves, and that with further reduction in the level of the nitrogen factor minimal light intensity would be associated with maximal growth.

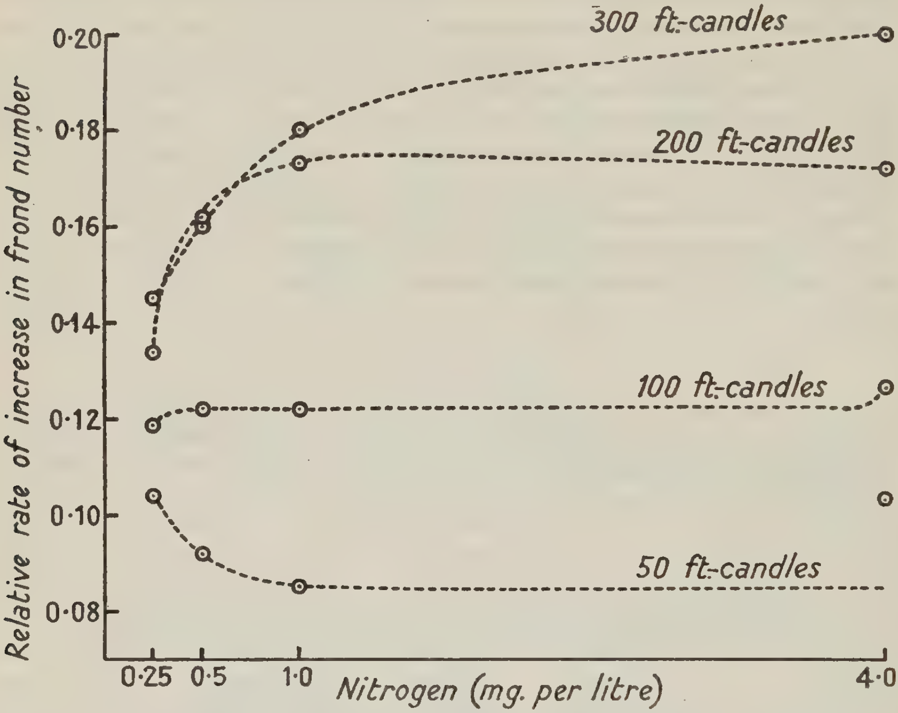

FIG. 1. Relative rate of increase in frond number plotted against nitrogen concentration for four levels of light intensity.

Frond area. The average frond area, with standard error for samples of eight plants of two fronds each, is given in Table II and the mean values for

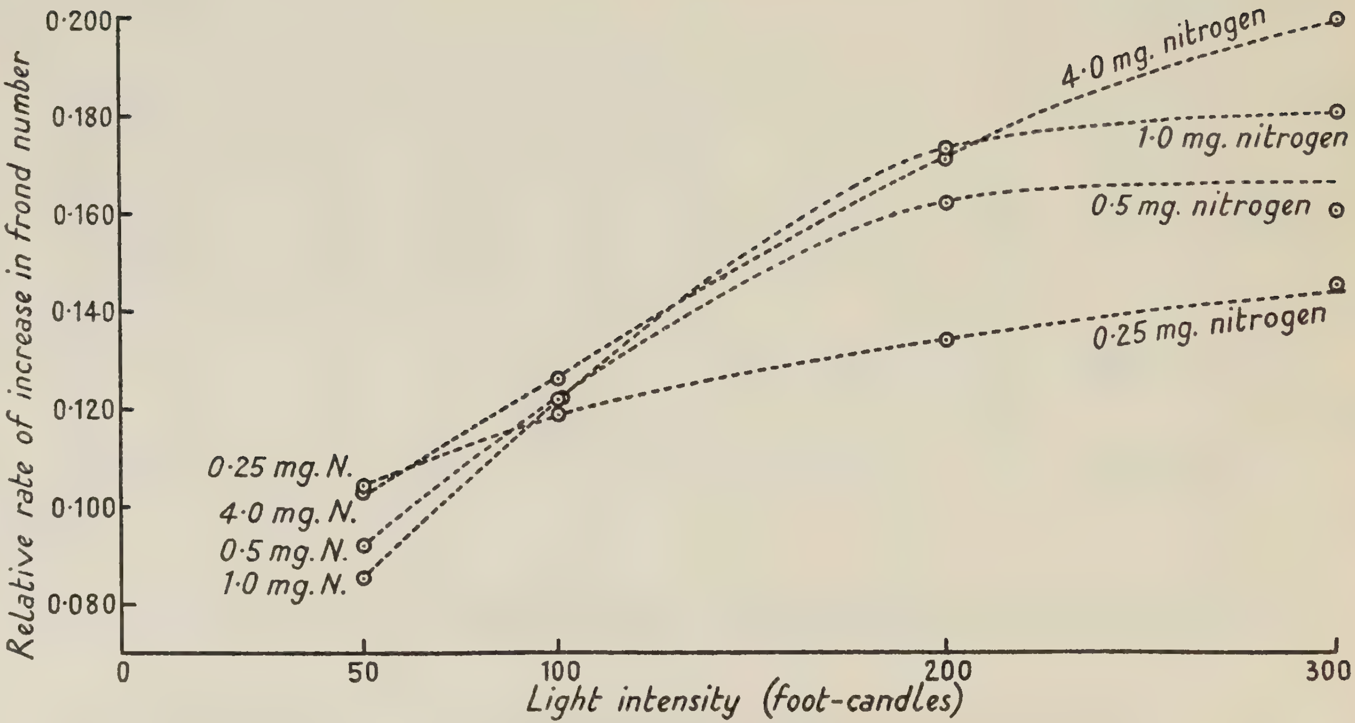

FIG. 2. Relative rate of increase in frond number plotted against light intensity for four levels of nitrogen concentration.

TABLE II

Average Frond Area (sq. mm. with S.E. of Samples) from 8th to 16th Day of Experiment of Colonies with Four Nitrate Concentrations and Four Light Intensities

Foot-candles:	300				200				100				50			
Nitrogen (mg. per litre)	4·0	1·0	0·5	0·25	4·0	1·0	0·5	0·25	4·0	1·0	0·5	0·25	4·0	1·0	0·5	0·25
Day 8 (29.7.32)	5·18 ±0·17	4·48 ±0·20	— —	— —	4·51 ±0·16	4·41 ±0·18	— —	— —	3·72 ±0·26	4·41 ±0·15	— —	— —	3·93 ±0·15	4·08 ±0·16	— —	— —
Day 9	— —	— —	4·36 ±0·24	3·92 ±0·15	— —	— —	4·28 ±0·18	4·20 ±0·14	— —	— —	4·28 ±0·18	4·15 ±·011	— —	— —	4·27 ±0·12	4·26 ±0·19
,, 10	5·13 ±0·10	4·44 ±0·25	— —	— —	4·45 ±0·13	4·35 ±0·10	— —	— —	4·37 ±0·11	4·07 ±0·25	— —	— —	3·92 ±0·18	3·89 ±0·26	— —	— —
,, 11	— —	— —	4·44 ±0·16	4·15 ±0·12	— —	— —	4·44 ±0·05	3·90 ±0·12	— —	— —	3·82 ±0·10	3·80 ±0·17	— —	— —	3·90 ±0·17	4·20 ±0·17
,, 12	4·95 ±0·11	4·97 ±0·12	4·14 ±0·10	3·71 ±0·13	4·37 ±0·13	4·09 ±0·12	3·90 ±0·04	3·46 ±0·11	— —	— —	— —	— —	— —	— —	— —	— —
,, 13	— —	— —	— —	— —	— —	— —	— —	— —	4·23 ±0·13	4·16 ±0·08	4·31 ±0·11	3·61 ±0·10	3·51 ±0·11	3·79 ±0·17	3·88 ±0·17	3·88 ±0·16
,, 14	5·11 ±0·12	4·78 ±0·15	3·79 ±0·12	3·79 ±0·18	4·37 ±0·06	4·54 ±0·13	3·96 ±0·14	3·81 ±0·09	— —	— —	— —	— —	— —	— —	— —	— —
,, 15	4·62 ±0·12	4·55 ±0·11	3·70 ±0·11	3·42 ±0·16	4·09 ±0·09	3·94 ±0·07	3·51 ±0·15	3·20 ±0·17	3·58 ±0·11	3·49 ±0·08	3·51 ±0·15	3·38 ±0·08	3·40 ±0·09	3·11 ±0·12	3·70 ±0·13	3·53 ±0·15
,, 16	4·67 ±0·14	4·03 ±0·13	3·16 ±0·10	2·88 ±0·13	3·67 ±0·08	3·54 ±0·10	3·42 ±0·15	2·61 ±0·11	— —	— —	— —	— —	— —	— —	— —	— —

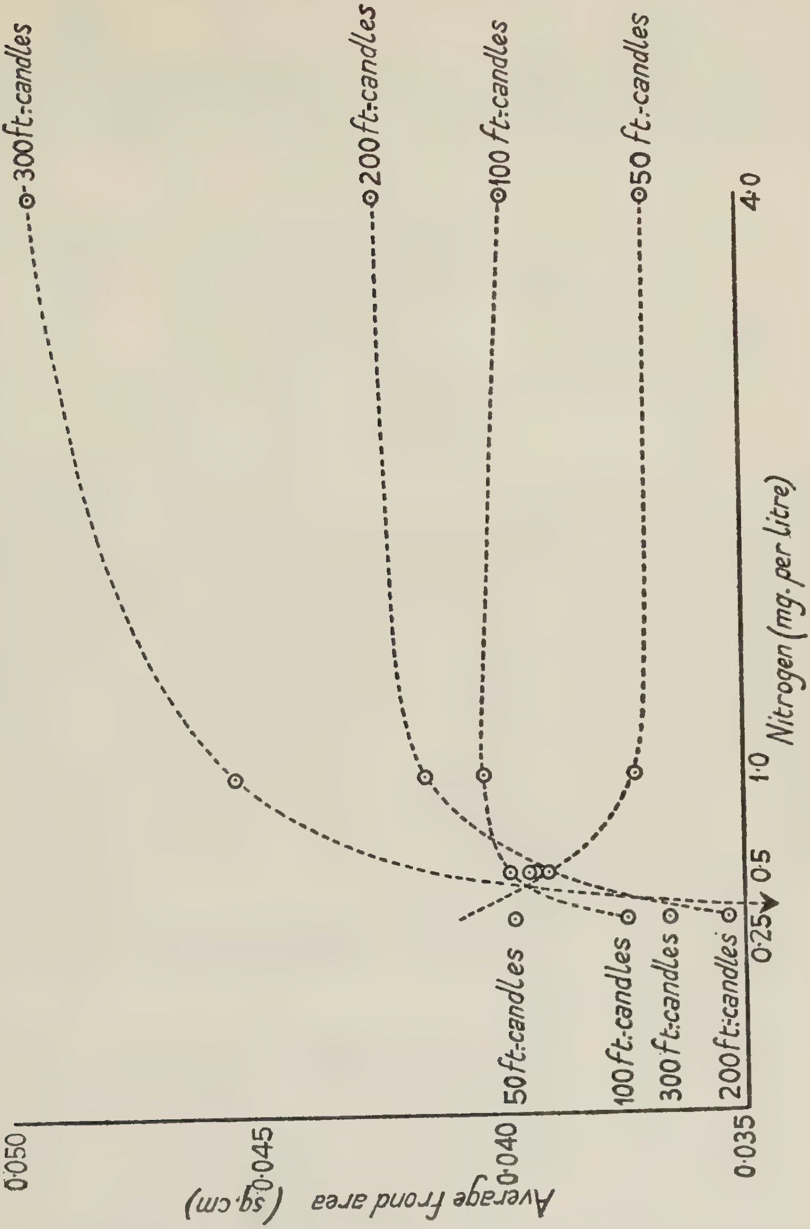

FIG. 3. Average frond area plotted against nitrogen concentration for four levels of light intensity.

each treatment are summarized in Table III. The effect of interaction between the factors may be studied in Figs. 3 and 4, which exemplify clearly the general inter-relationship between light intensity and nitrogen supply in the present series of experiments.

Attention may be directed first to Fig. 4, which shows the relation between frond area and light intensity for four levels of nitrogen supply.[1] At 300 ft.-candles (right-hand ordinate of Fig. 4) the colony with maximal frond area was obtained with a nitrogen concentration of 4 mg. per litre and with minimal frond area with 0·25 mg. per litre, the two intermediate concentrations giving intermediate values. On reduction of the light intensity to 50 ft.-candles (left-hand ordinate of Fig. 4) the reverse relation holds, for the plants of maximal area were obtained with 0·25 mg. nitrogen per litre and of minimal area with 4·0 mg. nitrogen per litre. According to the graph, maximal frond area between 300 and 160 ft.-candles is associated with a nitrate concentration equivalent to 4·0 mg. nitrogen per litre, between 160 and 90 ft.-candles with 1·0 mg. per litre, between 90 and 60 ft.-candles with 0·5 mg. per litre, and below 60 ft.-candles with 0·25 mg. per litre. Under a light intensity of 300 ft.-candles 0·25 mg. nitrogen per litre represents a 'limiting' concentration, but on reduction of the light factor to 50 ft.-candles this concentration becomes the most favourable. 4.0 mg. nitrogen per litre gives maximal area under 300 ft.-candles, but on reduction of the light factor to 50 ft.-candles this concentration is definitely detrimental. The optimal value of either nitrogen concentration or light intensity is clearly shown to vary with the level of the other factor.

TABLE III

Average Frond Area (sq. cm.) of Colonies with all Combinations of Four Nitrate Concentrations and Four Light Intensities

Light intensity.	Nitrogen (mg. per litre). 0·25.	0·5.	1·0.	4·0.
300 ft.-candles	0·0365	0·0393	0·0454	0·0494
200 ,,	0·0353	0·0390	0·0415	0·0424
100 ,,	0·0374	0·0398	0·0403	0·0398
50 ,,	0·0397	0·0394	0·0372	0·0369

Fig. 3 shows the relation between frond area and nitrogen supply for four levels of light intensity. The inter-relationship in Fig. 3 is the counterpart of that in Fig. 4, a region of relatively high nitrogen concentration ($>$1·0 mg. nitrogen per litre), where maximal light is associated with maximal frond area and minimal light with minimal frond area, being contrasted with a region

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[1] If a linear curve were drawn through the experimental points at levels of 4·0 and 1·0 mg. nitrogen per litre, the deviation of the experimental points from values calculated from the slope of the curve would be so great as to be beyond the range of experimental error. The type of curve drawn, which does not affect the conclusions as to the interaction of the factors, has been confirmed in later experiments at low light intensities.

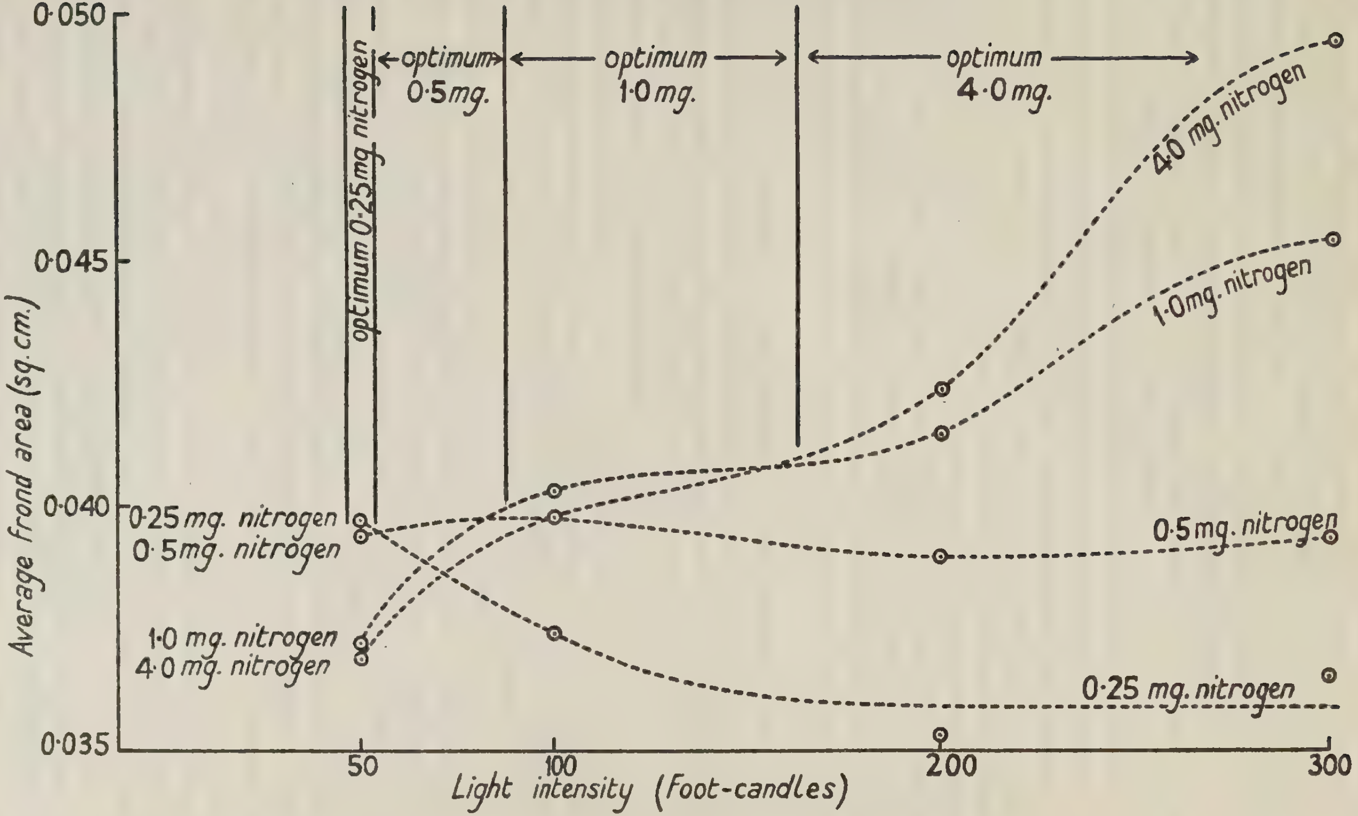

FIG. 4. Average frond area plotted against light intensity for four levels of nitrogen concentration.

of relatively low nitrogen concentration ($<0{\cdot}5$ mg. nitrogen per litre), where minimal light is associated with maximal frond area and, subject to experimental errors, maximal light with minimal frond area. At an intermediate concentration of about 0·5 mg. nitrogen per litre, where the curves intersect, the frond area of colonies under all light intensities is the same.

Fig. 3 brings out the fact that 4·0 mg. nitrogen per litre, a concentration scarcely sufficient to provide full growth under a light intensity of 300 ft.-candles, becomes supra-optimal when the light intensity is reduced to a very low level so that *reduction in nitrogen supply here leads to an increase in area.* Fig. 4 brings out the fact that 300 ft.-candles, a light intensity well below the optimum with adequate salt supply, becomes supra-optimal when the nitrogen supply is reduced low enough so that *reduction in light intensity here leads to an increase in area.*

Analysis of variance of frond area. In order to assess statistically the significance of these effects variance within colonies must be used as an estimate of error instead of variance between colonies, which is not available owing to lack of replication. Tests of significance of treatment would not appear to be invalidated by this procedure, provided the following conditions are satisfied.

(1) Variance between colonies is of the same magnitude as variance within colonies. At the end of the experiment the colony with maximal light intensity and full nitrogen supply was split into three sub-colonies which were grown under the experimental conditions in separate beakers for a period half as long as the experiment. Three samples for estimation of area were taken from each sub-colony. The variance of frond area of replicate samples from the same colony (S.D. = 5·9 per cent.) was found to be of the same order (no significant difference by Fisher's 'Z' test) as the variance of the frond area of replicate colonies (S.D. = 3·3 per cent.).

(2) Variance within colonies is independent of treatment. Inspection of the range of standard errors of Table II indicates that the frond areas of the nitrogen-starved colonies are somewhat more variable than the frond areas of the colonies with high nitrogen supply (readily apparent after conversion of the standard errors to a percentage basis). Increase of variance within colonies relative to variance between colonies would strengthen rather than vitiate treatment comparison based on the substitution of the former for the latter variance, since error would tend to be over-estimated in tests of significance.

An analysis of variance has therefore been carried out on the frond areas of Table II. In order to obtain a symmetrical table the values at 300 and 200 ft.-candles for days 14 and 16 are omitted, for the rate of increase in frond number at the lower light intensities had been insufficient to provide fronds for sampling on these days. There are 512 entries available made up of 4 light intensities × 4 nitrogen concentrations × 4 'occasions' of sampling (on most days half the treatments only are sampled so that two consecutive days are a single 'occasion') × 8 replicates to each sample. The 511 degrees of freedom consist of 9 representing the differences between the effects of light, nitrogen, and occasion of sampling; 54 representing the interaction between the variables; and a remainder of 448 representing the variability of the replicates within samples. For the method of calculation reference should be made to Fisher's 'Statistical Methods for Research Workers' (1934). The results are shown in Table IV, in which 'Z' values, representing the differences between the effects due to light, nitrogen, occasion of sampling, and their interactions and that due to replication within the samples are compared with 'Z' values corresponding with 5 per cent. and 1 per cent. probabilities. The separate effects of light, nitrogen, and occasion of sampling are seen to be highly significant. There is also a significant interaction of nitrogen and occasion, indicating that in some treatments high nitrogen supply is associated with constant frond area, and in others that frond area is falling in association with intensification of a starvation effect in the later stages of the experiment. The interaction of light and occasion is not significant, indicating that differences in frond area between the light treatments do not increase progressively with time. There is thus no evidence of an intensification of a carbohydrate starvation effect in the later stages of the experiment, and this leads to the view that the detrimental effect of decreasing light intensity on frond area is not due to progressively decreasing carbohydrate supply. The interaction of light and nitrogen is highly significant, and there is also a significant differential effect of occasion on this interaction.

TABLE IV

Analysis of Variance of Frond Area

	Degrees of freedom.	Variance.	'Z'.	1% point.	5% point.
Light	3	0·00076288	*1·8898*	0·6651	0·4787
Nitrogen	3	0·00047360	*1·6514*	0·6651	0·4787
Occasion	3	0·00093824	*1·9938*	0·6651	0·4787
Light × nitrogen	9	0·00018592	*1·1837*	0·4604	0·3309
Light × occasion	9	0·00002258	0·1296	0·4604	0·3309
Nitrogen × occasion	9	0·00003474	*0·3446*	0·4604	0·3309
Light × nitrogen × occasion	27	0·00004740	*0·5006*	<0·2000	<0·2000
Within samples	448	0·00001742	—	—	—

Net assimilation rate. Net increase in dry weight per unit area in unit time is estimated by $(W_2-W_1)\ (\log_e A_2-\log_e A_1)/A_2-A_1$ where A_1, A_2, W_1, W_2, are the total area and total dry weight of the colonies at times t_1 and t_2 respectively. The mean values for the experiment are given in Table V, which shows that net assimilation rate is influenced markedly by light intensity, whereas the effect of variation in nitrogen supply is slight.

Fig. 5, giving the relation between net assimilation rate and nitrogen concentration for four levels of light intensity, shows that for the two higher levels of light intensity there is a falling trend of net assimilation rate with decreasing nitrogen supply, whereas for the two lower levels of light intensity lower nitrogen concentrations tend to be associated with higher net assimilation rates.[1]

Fig. 6, showing the relation between net assimilation rate and light intensity for four levels of nitrogen supply, contains the curves for highest and lowest nitrogen concentrations only, for the points of the intermediate curves lie too close to be presented on the same diagram. Net assimilation rate is directly related to the light intensity between 50 and 300 ft.-candles and a linear curve has been fitted by the method of least squares to the experimental values for each level of nitrogen concentration. The experimental and calculated values are shown in Table VI. From the slopes of the curves the decrease in net assimilation rate per fall of 100 ft.-candles for different nitrogen concentrations may be obtained, and these values are given in Table VII. Since the maximal rate of fall is with maximal nitrogen concentration, and the minimal rate of fall with minimal nitrogen concentration, it is evident that with successive stages of decrease in light intensity the curves run together, intersect (at about 140 ft.-candles, Fig. 6), and then run apart so that *the effect of variation in nitrogen supply at high light intensity is reversed at low light intensity.*

TABLE V

Net Assimilation Rate (mg. per sq. cm. per two days) of Colonies with all Combinations of Four Nitrate Concentrations and Four Light Intensities. (Values in brackets represent mg. CO_2 *per sq. dm. per hour)*[2]

Light intensity.	Nitrogen (mg. per litre). 0·25.	0·5.	1·0.	4·0.
300 ft.-candles	0·543 (1·85)	0·557 (1·89)	0·584 (1·99)	0·617 (2·10)
200 „	0·394 (1·34)	0·405 (1·38)	0·414 (1·41)	0·418 (1·42)
100 „	0·268 (0·91)	0·280 (0·95)	0·243 (0·83)	0·234 (0·80)
50 „	0·176 (0·60)	0·143 (0·49)	0·122 (0·41)	0·145 (0·49)

It is of interest to note that exterpolated curves (represented by broken lines in Fig. 6) intersect the ordinate corresponding to zero light intensity at a level of at least 0·05 mg. per sq. cm. for two days. Direct proportionality between assimilation rate and light of very low intensity is not obtained.

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[1] In a previous communication (White, 1936*a*) it was shown that nitrogen starvation at a light intensity of 450 ft.-candles leads to *high* net assimilation rate, and this effect was attributed to *low* respiration rate, real assimilation being unaffected by nitrogen supply. Subsequent experiments have confirmed these effects, but suggest also that in a late stage of nitrogen starvation real assimilation falls, tending to reduce the net assimilation rate below that of a colony with full nutrient supply. The effect of nitrogen supply in the present experiment is conformable with this view, for during the first half of the experiment only the net assimilation rate of the colony with maximal nitrate concentration and high light intensity was 0·577 mg. per sq. cm. per two days in comparison with 0·644 mg. per sq. cm. per two days for the colony with minimal nitrate concentration and high light intensity.

[2] Conversion into carbon dioxide is based on the assumption that the composition of the dry frond material corresponds with the formula for starch or cellulose.

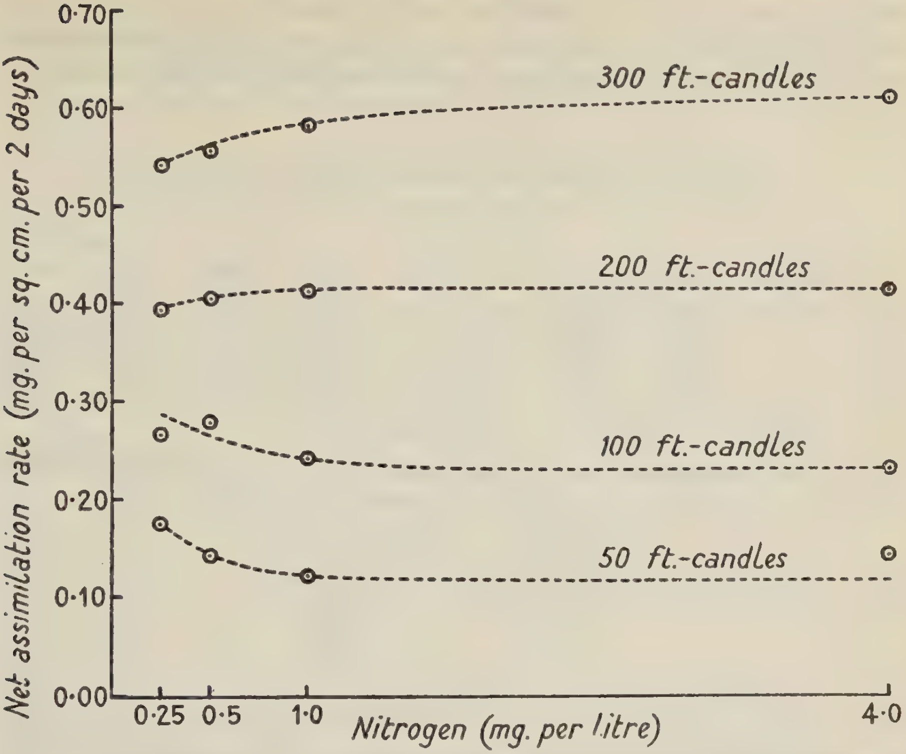

FIG. 5. Net assimilation rate plotted against nitrogen concentration for four levels of light intensity.

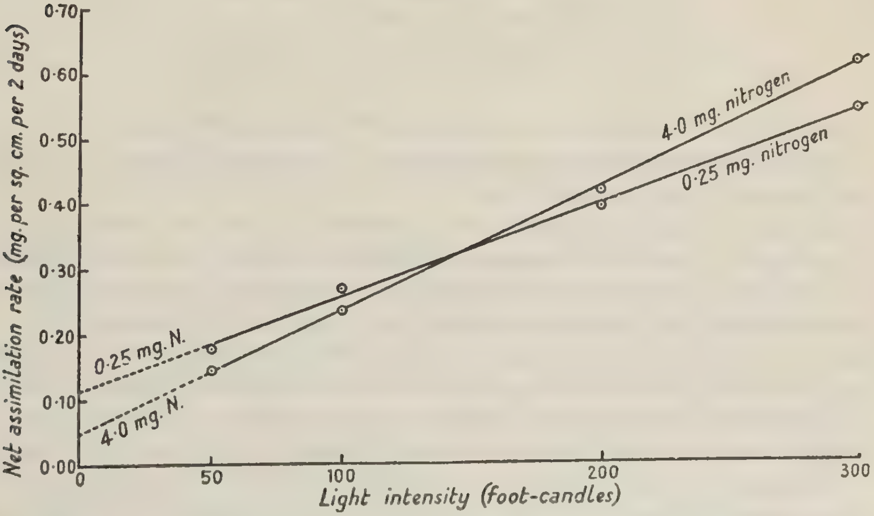

FIG. 6. Net assimilation rate plotted against light intensity for two levels of nitrogen concentration.

Dry weight per unit area. Mean dry weight per unit area is shown in Table VIII and represented graphically in Fig. 7, which shows the relation between dry weight and nitrogen concentration for four levels of light intensity and

Table VI

Calculated and Experimental Values of Net Assimilation Rate (mg./sq. cm./two days)

Nitrogen (mg. per litre).	0·25.		0·5.		1·0.		4·0.	
	calc.	exp.	calc.	exp.	calc.	exp.	calc.	exp.
50 ft.-candles	0·184	0·176	0·169	0·143	0·137	0·122	0·142	0·145
100 ,,	0·255	0·268	0·248	0·280	0·227	0·243	0·236	0·234
200 ,,	0·398	0·394	0·406	0·405	0·408	0·414	0·425	0·418
300 ,,	0·541	0·541	0·564	0·557	0·589	0·584	0·614	0·617

Table VII

Fall in Net Assimilation Rate (mg./sq. cm./two days) Corresponding to Decrement of 100 ft.-candles

Nitrogen (mg. per litre).	Mg. per sq. cm. per two days.
4·0	0·189
1·0	0·181
0·5	0·158
0·25	0·143

Table VIII

Dry Weight per Unit Area (mg. per sq. cm.) of Colonies with Four Nitrate Concentrations and Four Light Intensities

Light intensity.	Nitrogen (mg. per litre) 0·25.	0·5.	1·0.	4·0.
300 ft.-candles	2·12	1·90	1·64	1·57
200 ,,	1·68	1·47	1·33	1·27
100 ,,	1·05	1·02	1·03	1·02
50 ,,	0·96	1·00	1·00	0·99

Fig. 8, which shows the relation between dry weight and light intensity for four levels of nitrogen supply.

At a level of 300 ft.-candles in Fig. 7 there is appreciable increase in dry weight per unit area with reduction in nitrogen supply. The association of high dry weight and low nitrogen supply is less accentuated at 200 ft.-candles, and at 100 and 50 ft.-candles variation in nitrogen supply has no effect. All four curves relating dry weight and light intensity in Fig. 8 are of sigmoid type. As the light intensity is reduced from 100 to 50 ft.-candles these curves tend to become superimposed, indicating that at very low light intensity dry weight is not affected by variation in either light intensity or nitrogen supply.

Comparison of Fig. 7 with Fig. 1 shows that at light intensities of 300 and 200 ft.-candles dry weight per unit area is inversely related to rate of increase in frond number. As the rate of frond multiplication falls there is evidently an accumulation of carbohydrates, for net assimilation is not appreciably affected by decreasing nitrogen supply (Table V). At 100 ft.-candles variation in nitrogen supply has no effect, either on rate of increase in frond number or on weight per unit area. Further reduction in light intensity to 50 ft.-candles *reduces appreciably the rate of increase in frond number* (*Fig. 1*) *but does not affect dry weight* (*Fig. 7*). Dry weight per unit area may be considered a measure of the accumulated carbohydrate over and above that used in growth. The fact that dry weight per unit area remains constant with falling light in spite of a steady fall in net assimilation demonstrates that at very low light intensity the rate of frond multiplication is not controlled by the carbohydrate level. The inference that frond area under very low light intensity is not closely related to the carbohydrate level (p. 633) supports this interpretation of the results. Further confirmation of this point is provided by the curves of daily counts of frond number at low light intensity, for these plotted on a logarithmic scale are linear to the end of the experiment, giving no indication of progressive carbohydrate starvation.[1]

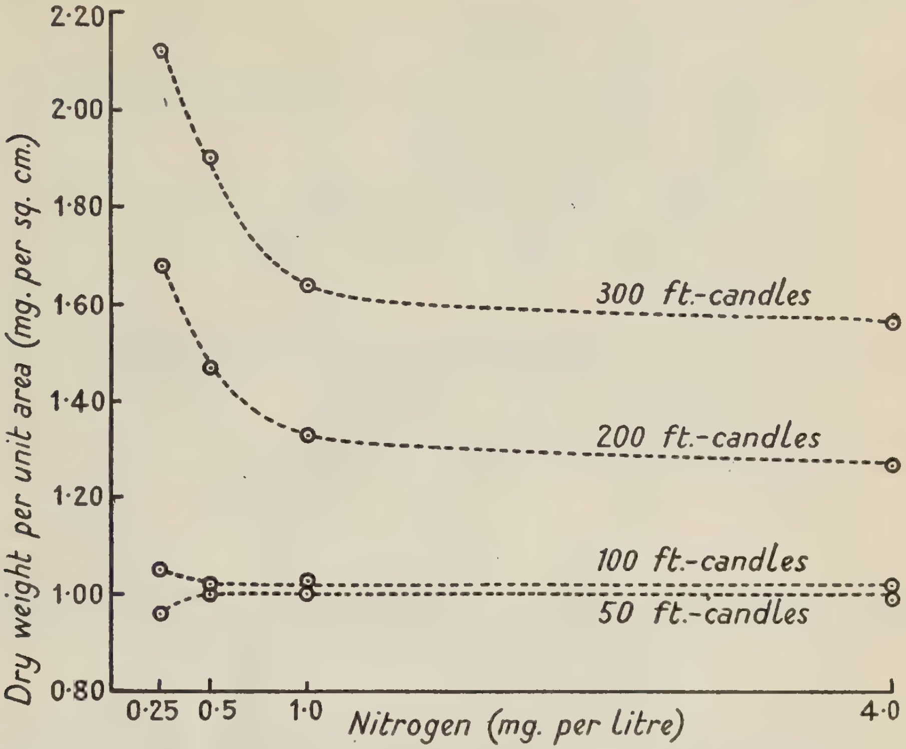

FIG. 7. Dry weight per unit area plotted against nitrogen concentration for four levels of light intensity.

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[1] Additional evidence of the independence of growth and carbohydrate supply at very low light intensity has been obtained in later experiments.

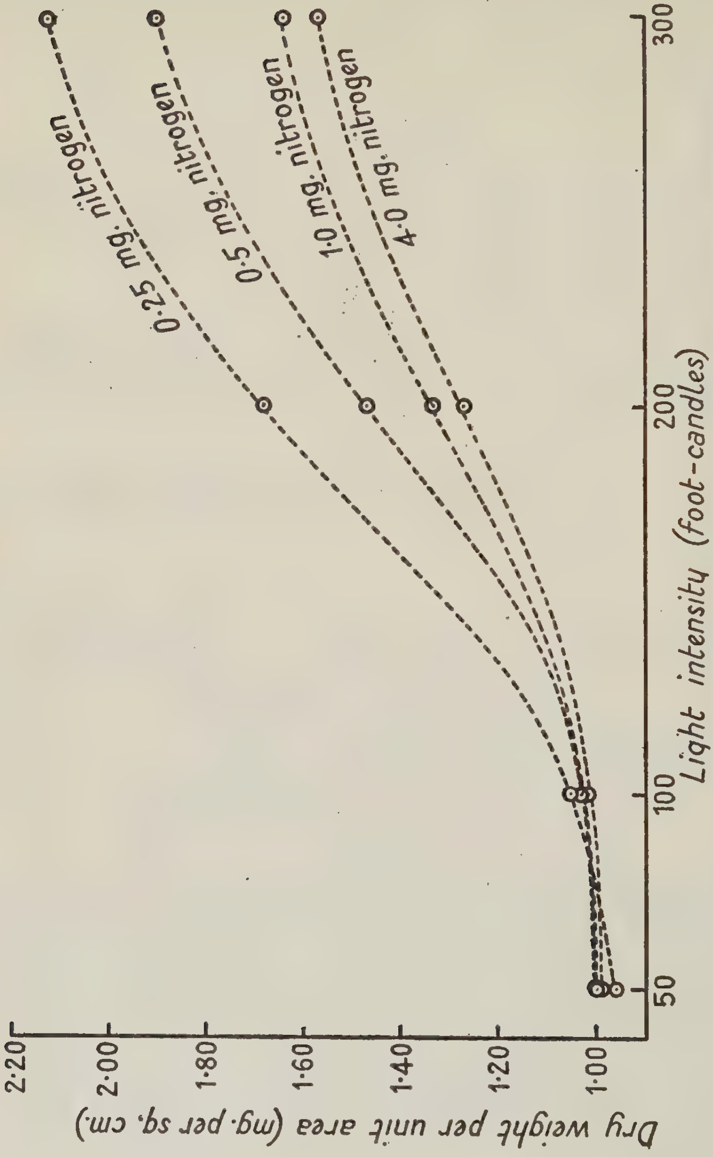

Fig. 8. Dry weight per unit area plotted against light intensity for four levels of nitrogen concentration.

Starch and protein content and depth of frond colour. Tests for starch and protein by plunging in hot water, decolorizing in warm alcohol, and staining whole fronds with iodine and Millon's reagent were carried out on samples from all treatments at the end of the experiment. Relative differences in depth of frond colour were noted daily from the samples taken and the results collected and averaged at the end of the experiment. These results are all expressed on arbitrary scales from 0 (no starch, no protein, frond colour very pallid) to 10 (dense starch, dense protein, deep green frond colour). All differences were checked independently by observers who did not know the combinations of treatment applied. It is assumed that differences of 3 on these scales exceed the range of error of estimation and therefore represent real effects, while intermediate values between the colonies at each end of a series are confirmatory.

TABLE IX

Relative Starch Content (Iodine Colour Test) of Colonies with all Combinations of Four Nitrate Concentrations and Four Light Intensities

Light intensity.	Nitrogen (mg. per litre).			
	0·25.	0·5.	1·0.	4·0.
300 ft.-candles	10	10	9	7
200 ,,	9	7	5	4
100 ,,	4	3	2	2
50 ,,	2	1	1	1

Relative Protein Content (Millon's Colour Test) of Colonies with all Combinations of Four Nitrate Concentrations and Four Light Intensities

Light intensity.	Nitrogen (mg. per litre).			
	0·25.	0·5.	1·0.	4·0.
300 ft.-candles	2	3	6	7
200 ,,	4	5	5	6
100 ,,	5	6	5	4
50 ,,	6	6	5	3

Relative Depth of Frond Colour of Colonies with all Combinations of Four Nitrate Concentrations and Four Light Intensities

Light intensity.	Nitrogen (mg. per litre).			
	0·25.	0·5.	1·0.	4·0.
300 ft.-candles	2	3	5	8
200 ,,	3	4	7	6
100 ,,	5	6	5	4
50 ,,	5	5	4	3

The results are given in Table IX and shown diagrammatically in Figs. 9, 10, and 11. Fig. 9 (starch content) resembles closely Fig. 7 (dry weight per unit area) whereas Fig. 10 (protein by Millon's test) and Fig. 11 (depth of frond colour) resemble Fig. 2 (average frond area). The correlation coefficients between these pairs of variates over the sixteen combinations of light intensities and nitrogen concentrations studied are as follows:

	r.	n.	P.
Dry weight per unit area and starch content	+0·958	14	<0·01
Average frond area and protein content	+0·672	14	<0·01
Protein content and depth of frond colour	+0·764	14	<0·01

Starch content and dry weight per unit area approximate so closely that they may be considered directly proportional. It is of interest to note that starch was detected in fronds from all treatments, even those with light intensity as low as 50 ft.-candles. Protein content by Millon's test and average frond area are significantly correlated as is depth of frond colour.

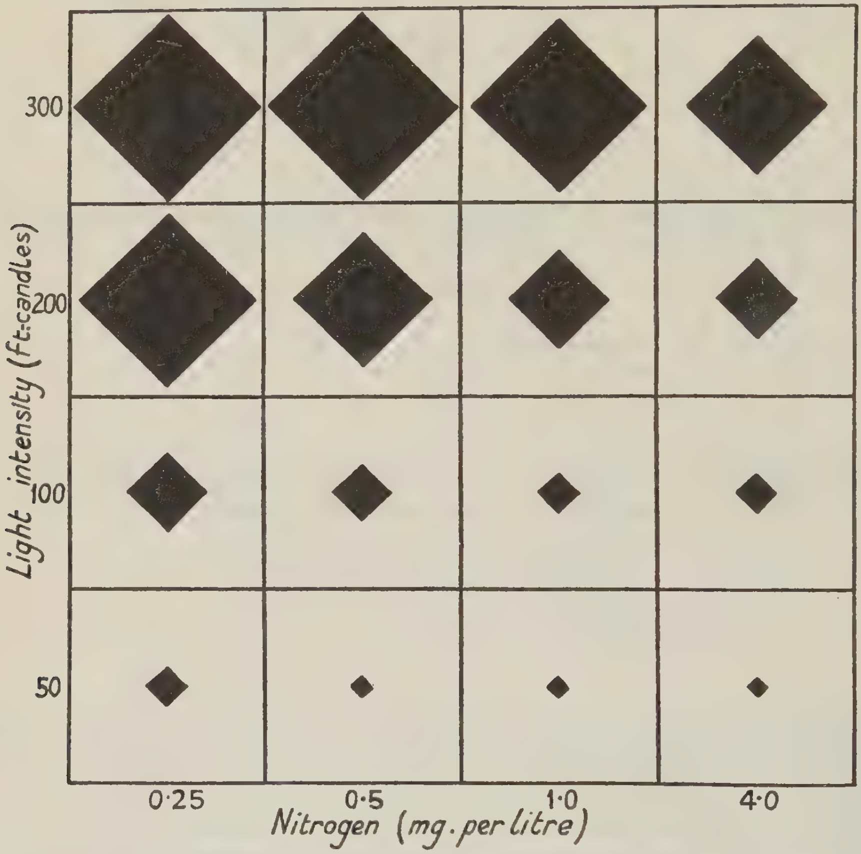

FIG. 9. The areas of the black squares give a measure of the relative starch content, estimated from the intensity of the iodine reaction, for combinations of four light intensities and four nitrogen concentrations.

With high nitrogen supply, reduction in intensity of light has nearly as marked an effect upon decrease in protein content as has nitrogen starvation under high light intensity (Fig. 10). But if the nitrogen content of the solution is reduced from 4·0 to 0·25 mg. per litre then *reduction in light intensity is associated with an increase in protein content.* And if the light intensity is reduced from 300 to 50 ft.-candles *decrease in nitrogen content is associated with increase in protein content.* These differences were clear-cut and confirmed by competent observers, independently of the writer.

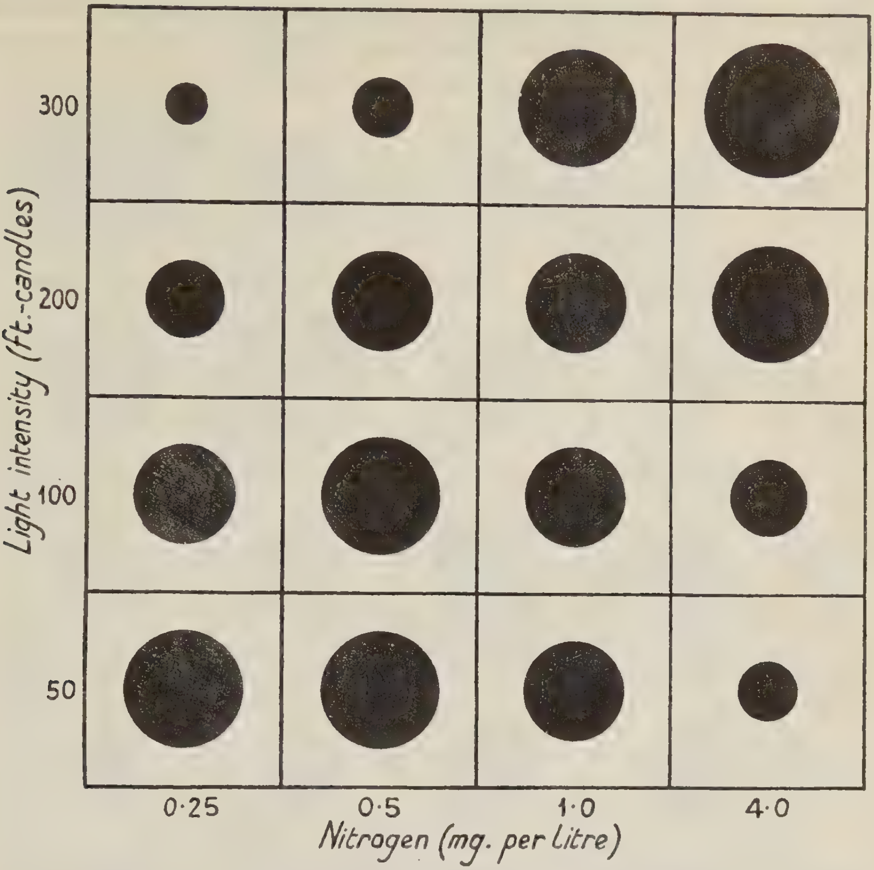

FIG. 10. The areas of the black circles give a measure of the relative protein content, estimated from the intensity of Millon's colour test, for combinations of four light intensities and four nitrogen concentrations.

III. MODE OF INTERACTION OF NITROGEN AND LIGHT

Examination of the tables and diagrams shows that at *each* level of light intensity the plant colony, characterized by greatest depth of frond colour, highest protein content, largest frond area, and probably most rapid rate of increase in frond number, tends to occur in a *different* nitrogen concentration. Similarly, for *each* level of nitrogen concentration the colony with maximal growth values tends to occur under a *different* light intensity. The treatments may be arranged in the form of a square, as shown in Fig. 12, with two diagonally opposite corners (upper right and lower left) representing deep frond colour, high protein content, &c., and two diagonally opposite corners representing the reverse symptoms. A diagonal line represents maximal growth values for each level of either factor. The experiment demonstrates clearly that nitrogen starvation may be induced either by increasing the light intensity or by decreasing the nitrogen supply. Growth, free from starvation symptoms, is obtained only when the levels of the two factors are in a suitable ratio. To retain this condition of 'balance', illustrated by the course of the diagonal line of Fig. 12, increase or decrease of one factor must be accompanied by corresponding increase or decrease of the second factor.

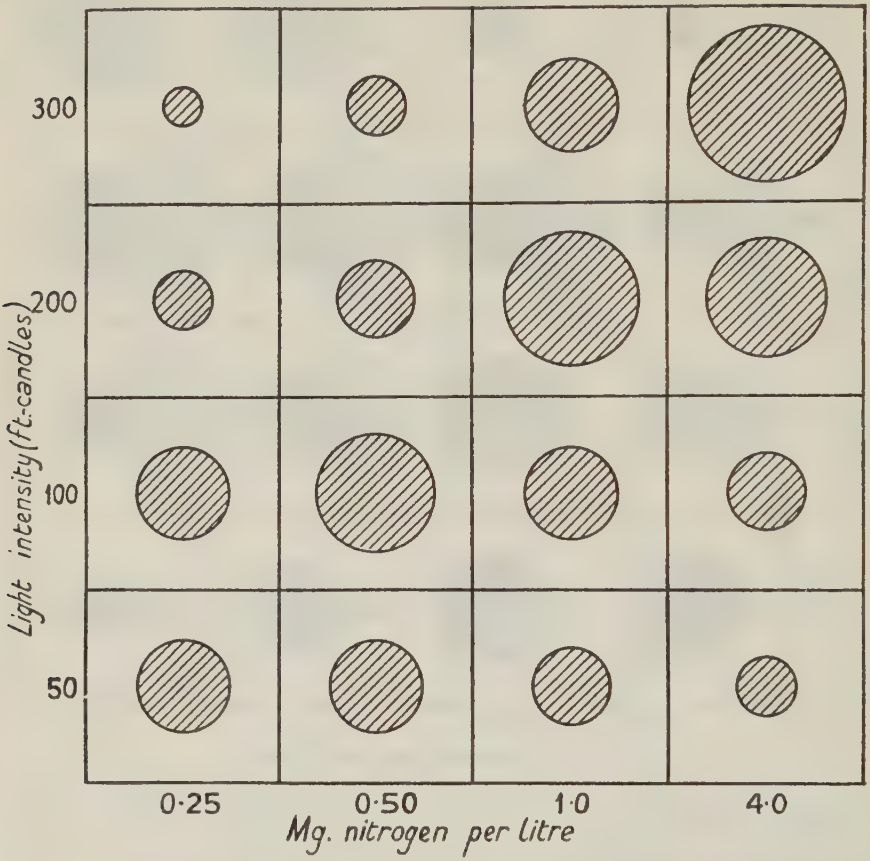

FIG. 11. The areas of the shaded circles give a measure of the relative depth of frond colour for combinations of four light intensities and four nitrogen concentrations.

In order to throw light upon the nature of this 'balance', reference may be made to Fig. 10, giving the results of tests for protein. Starting from the colony under maximal light intensity of 300 ft.-candles and with maximal nitrogen concentration of 4·0 mg. per litre (top right-hand corner in Figs. 10

and 12), reduction in nitrogen concentration leads to less protein content. But the low protein content of this colony (top left-hand corner in Figs. 10 and 12), together with all other symptoms of nitrogen starvation, such as high dry weight per unit area and excess starch content, is alleviated by decrease of light intensity. Now the protein content as estimated *is not the protein formed but the difference between that formed and that consumed in the building of new tissue and in the formation of new fronds.* The rate of frond number increase of the colony with low nitrogen but high light, represented by an exponential index of 0·145, is considerably higher than the rate of frond number increase —0·104—of the colony with low nitrogen but low light. The demand for protein for formation and growth of new fronds was correspondingly higher and, since both colonies had an equal nitrogen supply, that under the highest light intensity must be expected to be subject to greater relative nitrogen deficiency. It is held that the effect of light upon rate of frond number increase, an effect which is exerted *in spite of variation in nitrogen concentration*, leads under higher light to higher protein demand, higher consumption, and a more acute condition of internal nitrogen starvation.

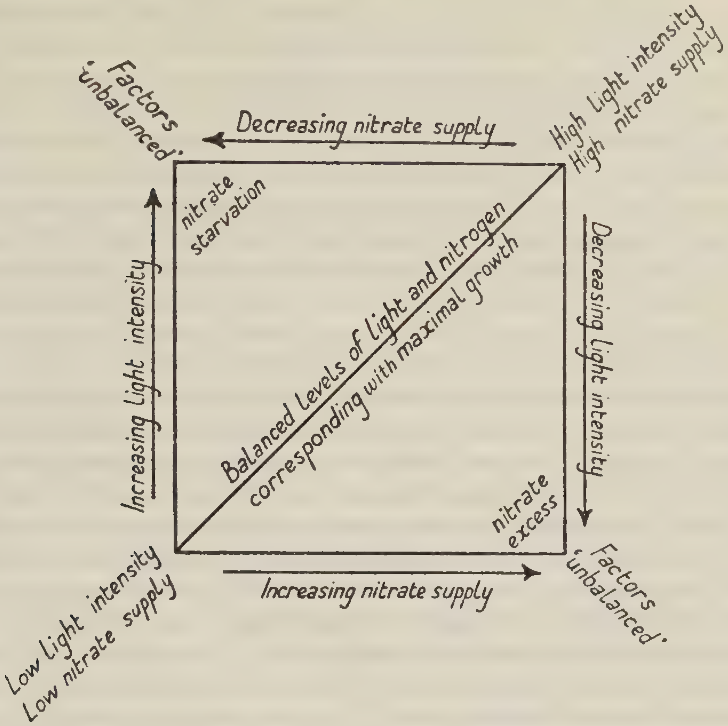
'Factors unbalanced'
Decreasing nitrate supply
High light intensity
High nitrate supply
nitrate starvation
Increasing light intensity
Balanced levels of light and nitrogen
corresponding with maximal growth
Decreasing light intensity
nitrate excess
Low light intensity
Low nitrate supply
Increasing nitrate supply
'Factors unbalanced'

FIG. 12. Theoretical representation of interaction between nitrogen supply and light intensity. The diagonal line represents maximal growth for each level of either factor.

Turning from the colony under minimal light intensity of 50 ft.-candles and with minimal nitrate concentration of 0·25 mg. per litre (bottom left-hand corner of Figs. 10 and 12) to the colony under the same light intensity but with maximal nitrate supply of 4·0 mg. per litre (bottom right-hand corner of Figs. 10 and 12), it is seen that *increase in nitrate supply leads to the apparently anomalous effect of a decrease in protein content.* This effect is not due to change in rate of frond multiplication, which does not differ markedly in the series under minimal light intensity. It has been shown that the growth data are not conformable with the view that at low light intensity growth is limited by carbohydrate level and therefore the hypothesis must be rejected that low light intensity leads to low protein content because light is necessary for the formation of sugars, which are essential for the construction of proteins. Nor can a low protein content at 50 ft.-candles be attributed to a direct formative effect of light on protein synthesis, since in the series under consideration the light intensity is constant, whereas the protein content shows wide variation. The conclusion is drawn that *it is the increase in nitrate supply that is responsible for the reduction in protein content.* It appears that the curve relating protein content and nitrate supply has a well-defined optimal point and that with nitrate values above and below this point there is a marked falling off in protein content and in growth values correlated with protein content.[1]

The decrease in relative protein content with increase in light intensity at the minimal nitrogen value of 0·25 mg. per litre (Fig. 10) has been attributed to a concomitant increase in rate of frond multiplication, leading to greater protein consumption with equal nitrogen supply. But should the nitrogen concentration be increased to its maximal value of 4·0 mg. per litre then *decrease in light intensity must decrease protein content, for at low light intensity this concentration is above the optimum for protein content.* As the light intensity is reduced from 300 to 50 ft.-candles the index of relative rate of increase in frond number falls from 0·199 to 0·103. The demand for nitrogen for the formation of new fronds also falls, so that there must be reduced nitrogen consumption and an accumulation of nitrate in the fronds of the colony under low light but high nitrate supply. *It is the relative increase in the nitrate factor rather than a direct reduction in the intensity of the light factor that is held responsible for the falling protein content.*[2] In the same way as the symptoms of nitrogen starvation are accentuated by increase in intensity of the light factor, which increased growth *in spite of a supposedly 'limiting' effect of low nitrogen supply*, so are the symptoms of nitrate excess accentuated by a decrease in

[1] There is a striking resemblance between these results with Lemna under continuous illumination of low intensity and those of Nightingale (1927) for Salvia growing in short days in which there was accumulation of nitrate in association with relatively high carbohydrate level and a low protein level.

[2] In order to elucidate the effect of light on protein content it is essential to vary the nitrogen supply as the light intensity is reduced so that these factors remain in an approximately similar ratio. Differences between protein contents corresponding to such 'balanced' combinations (Fig. 12) fail to reach a significant level (Table IX and Fig. 10) suggesting that in this experiment protein synthesis is not affected *directly* by the intensity of light.

the intensity of the light factor which reduces growth *in spite of an apparent detrimental effect of excess nitrate concentration.*

The considerations outlined show how on the one hand growth characters associated with a decrease in the nitrate factor are attained either by a direct reduction in the nitrate supply or by increase in the light intensity and how, on the other hand, growth characters associated with increase in the nitrate factor are attained either by a direct increase in nitrate supply or by a decrease in light intensity. Whatever the level of a factor, that factor alone cannot be considered to control the rate of growth, which is determined rather by a combination of both factors. The diagonal line of Fig. 12 represents 'balanced' values of the factors corresponding to maximal growth, and it is patent that a scheme of the effect on growth of all combinations of these two factors must show such a diagonal representing at different levels the optimal combinations of the two factors.

IV. General Theory of Interaction between Factors

The phenomena discussed suggest a theory of interaction between factors that may be stated in general terms. According to this theory, the effect of increase of a factor is to increase the rate of one or many physiological processes, referred to as 'growth', and thereby create increased demand for other factors. Should any of these factors be present at low level, symptoms of starvation due to their deficiency occur, for the same supply has now to satisfy a larger demand. Whether increased growth actually occurs or not is the resultant of a positive effect due to the increase of the major factor and a negative effect due to the greater relative deficiency of all other factors and is determined in any particular case by the relative importance of the factors at work. The effect of decrease of a factor is similar in principle but works in the opposite direction, i.e. consequent decreased growth is associated with a relative excess of any other factors contributing to growth, that were previously present in sufficiency. Should any of these factors be harmful in excess supra-optimal symptoms appear.

V. Summary

1. A study has been made under controlled conditions of the effect on the growth and assimilation of *Lemna minor* of all combinations of four nitrate concentrations (4·0, 1·0, 0·5, and 0·25 mg. nitrogen per litre) and four light intensities (300, 200, 100, and 50 ft.-candles).

2. The optimal nitrate concentration varies with the light intensity, rising with increasing light intensity and falling with decreasing light intensity. The optimal light intensity also rises and falls with the level of the nitrogen factor.

3. At the highest light intensity studied decreasing nitrogen supply is associated with lower rate of increase in frond number, lower net assimilation rate, smaller frond area, less protein content, and a loss of chlorophyll. At

the lowest light intensity studied decreasing nitrogen supply is associated with higher rate of increase in frond number, higher net assimilation rate, larger frond area, greater protein content, and deeper frond colour. The trend of growth with decreasing light intensity at high nitrogen concentration is reversed at low nitrogen concentration.

4. The characteristic symptoms of nitrogen deficiency are intensified by increase of light intensity with constant nitrogen supply, and those of nitrate excess by decrease of light intensity with constant nitrate supply.

5. Although a linear relation between net assimilation rate and light intensity of between 300 and 50 ft.-candles shows that the rate of photosynthesis is determined directly by light at intensities of this order, the rate of frond multiplication at the minimal experimental light intensity (50 ft.-candles) is not controlled by the low carbohydrate supply resulting from the reduced photosynthetic rate. Reduction in light intensity from 100 to 50 ft.-candles does not reduce the dry weight per unit area and growth at very low light intensity is not controlled by carbohydrate level.

6. Significant correlation coefficients for the sixteen combinations of light intensity and nitrogen concentration are found between dry weight per unit area and starch content ($r = +0{\cdot}958$), frond area and protein content ($r = +0{\cdot}672$), and protein content and depth of frond colour ($r = +0{\cdot}764$).

7. The interaction between nitrogen and light is held to be explicable on the following basis. Variation in nitrogen supply affects but slightly the accelerating effect of light upon rate of increase in frond number, so that the rate of protein consumption in the formation of new fronds is higher under high light intensity and, with low nitrate supply, leads to a more acute condition of internal nitrogen starvation. Variation in nitrogen supply affects but slightly the retarding effect of decreasing light intensity on the rate of increase in frond number, so that the consumption of nitrogen in the formation of new fronds falls off under low light intensity and leads to an intensification of the symptoms of an excess of nitrates in the fronds of the colony with high nitrate supply

8. It is concluded that maximal growth is obtainable only when the levels of the factors of light and nitrogen are in a suitable ratio. To retain this condition of 'balance', increase or decrease of one factor must be accompanied by corresponding increase or decrease of the other factor. A general theory, based on this principle, of the mode of interaction between factors is put forward.

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The Interaction of Factors in the Growth of Lemna

XII. The Interaction of Nitrogen and Light Intensity in Relation to Root Length

BY

H. L. WHITE, PH.D.

(From the Research Institute of Plant Physiology, Imperial College of Science and Technology, London)

With two Figures in the Text

The root of Lemna minor. Each single plant of *Lemna minor*, consisting of three fronds or less, possesses a single unbranched root, provided with a prominent root-cap. The root probably functions both as an absorbing organ and as an anchor. On the approach of senescence the root-cap is lost, starch disappears, and the root becomes turned up at the end and wrinkled. Roots are normally colourless but may under certain conditions, which have not yet been fully explored, develop chlorophyll.

TABLE I

Mean Length of Root (cm.) with S.E. of Samples of 10 Plants of Approximately Equivalent Age

Light intensity (ft.-candles).	With full nutrient supply.	With nitrogen deficiency.	Percentage increase.
1200	3·30±0·15	4·45±0·09	34·8
900	2·78±0·18	3·93±0·12	41·4
600	1·97±0·09	3·06±0·10	55·3
300	1·01±0·19	2·08±0·08	105·9

Experimental results. Attention was first directed to a relationship between the metabolism of the plant and root development by an experiment in which nutrient solution circulated continuously through four growth chambers containing four Lemna colonies, each under a different intensity of light. The effect upon root-length of reduction in nitrate supply to moderate starvation level is seen in Table I, which shows that with low nitrogen supply the roots of the colony under a light intensity of 300 ft.-candles increased in length by as much as 105 per cent., whereas the roots of the colony under a light intensity of 1,200 ft.-candles increased only by 35 per cent. Increase in root-length is characteristic of moderate nitrogen starvation (White, 1936, Table III), and it is evident that the intensity of this effect is profoundly influenced by the level of light intensity.

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Measurements of root-length were made in an experiment in which Lemna colonies were subjected to all combinations of four light intensities—300, 200, 100, and 50 ft.-candles—and four nitrate concentrations—equivalent to 4·0, 1·0, 0·5, and 0·25 mg. nitrogen per litre. Reference should be made to a separate paper of this series (White, 1937) for details of the technique and growth data obtained. The mean root-lengths of samples of eight plants of approximately equivalent age are given in Table II. From the range of standard errors of the means it is apparent that variation in both light intensity and nitrogen supply has a highly significant effect on length of root; and this effect is not the same for one level of either factor as for a different level. The sixteen samples were dried under standard conditions in vacuo according to the method of Su and Ashby (1929) and weighed on a microchemical balance. The dry weight of the roots could not conveniently be estimated separately and was included in the total dry weight of the plants, but its percentage contribution to the total dry weight is of the order of 14 per cent.,[1] so that differences in degree of root development cannot have materially affected the weight, which was virtually that of the fronds. The mean dry weight per plant of two fronds, i.e. one which bears a single root, is given in Table III.[2] The ratio between dry weight and root-length, which is taken as a measure (inverse) of relative root development, is given in Table IV and Fig. 1.

TABLE II

Mean Length of Root (cm.) with S.E. of Samples of 8 Plants of Approximately Equivalent Age

Light (ft.-c.).	0·25	0·5	1·0	4·0	N (mg. per litre).
300	2·8±0·14	2·7±0·09	2·2±0·07	1·7±0·05	
200	2·1±0·17	1·6±0·06	1·4±0·09	1·0±0·05	
100	0·6±0·03	0·5±0·04	0·5±0·02	0·4±0·03	
50	0·3±0·04	0·3±0·04	0·3±0·03	0·3±0·04	

TABLE III

Dry Weight (mg.) per Plant of 2 Fronds

Light (ft.-c.).	0·25	0·5	1·0	4·0	N (mg. per litre).
300	0·150	0·154	0·166	0·154	
200	0·112	0·108	0·112	0·108	
100	0·068	0·070	0·072	0·070	
50	0·064	0·078	0·066	0·070	

Chemical analyses of these plants are not available, but an estimate of the relative starch and protein contents was obtained by plunging samples into hot water, decolorizing in warm alcohol, and staining with dilute iodine and Millon's reagent respectively (White, 1937). The effects of the treatments on the *ratios* between starch and protein are shown in Table V and Fig. 2, which should be contrasted with Fig. 1. It is strikingly evident that in whichever direction a line is drawn across one of these diagrams ascending or descending values are accompanied by converse descending or ascending values on the complementary diagram. That these relations are quantitative is demonstrated by measurement of their intensity by calculation of a correlation coefficient of −0·693 which corresponds with a probability of 0·01 (Fisher, 1934). Relative root development is therefore correlated with the starch-protein ratio. Although it cannot be determined that 'starch' and 'protein', as estimated, are representative of the general carbohydrate-nitrogen balance of the plants it seems justifiable to assume, in view of the low light intensities (<300 ft.-candles) and low nitrate concentrations (<4·0 mg. nitrogen per litre) used in the experiment, that such was the case. If the increase in length of root with increase in light intensity or decrease in nitrogen supply is due to increase in carbohydrate level relative to protein content, then any factor

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[1] I am indebted to Mr. T. A. Oxley for this determination.

[2] The data of Table III confirm the fact that variation in nitrogen supply does not affect appreciably the frond dry weight (White, 1936, Table I), the decrease in area associated with nitrogen deficiency being roughly of the same magnitude as increase in dry weight per unit area.

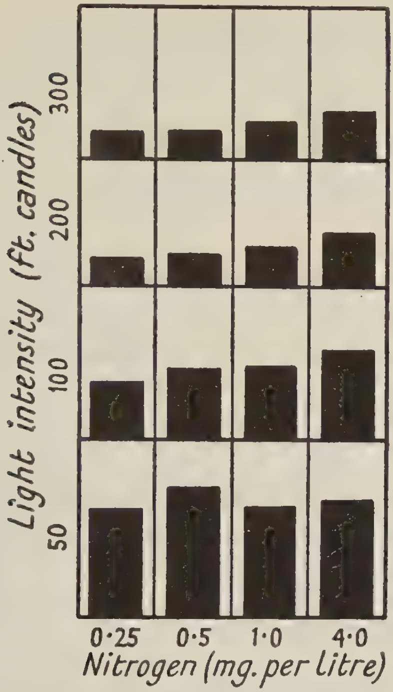

FIG. 1. The heights of the columns give a measure of the ratio of dry weight (mg.) to length of root (cm.) of Lemna colonies subjected to sixteen combinations of light intensity and nitrogen supply.

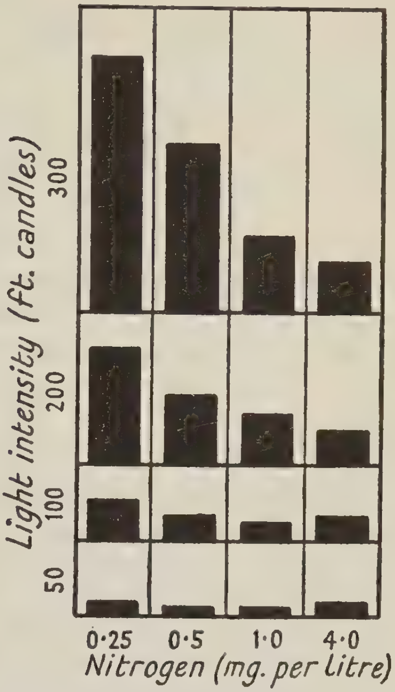

FIG. 2. The heights of the columns give a measure of the ratio of starch to protein of Lemna colonies subjected to sixteen combinations of light intensity and nitrogen supply.

that raises the assimilation rate would tend to produce the same effect, i.e. a colony with exceptionally long roots. It should be possible to confirm this point by raising the level of the CO_2 factor, and the author is indebted to

TABLE IV

Ratio ($\times 10^3$) of Dry Weight of Fronds (mg.) to Length of Root (cm.)

Light (ft.-c.).	0·25	0·5	1·0	4·0 N (mg. per litre).
300	54	57	75	91
200	53	67	80	108
100	113	140	144	175
50	213	260	220	233

TABLE V

Ratio of Starch to Protein as Estimated from Staining with Iodine and Millon's Reagent

Light (ft.-c.).	0·25	0·5	1·0	4·0 N (mg. per litre).
300	5·0	3·3	1·5	1·0
200	2·3	1·4	1·0	0·7
100	0·8	0·5	0·4	0·5
50	0·3	0·2	0·2	0·3

Dr. P. Hicks for the communication (unpublished) that one of the most noteworthy features of Lemna colonies grown in atmospheres enriched with CO_2 is the striking development of the roots.

On the mechanism of the relation between carbohydrate-nitrogen balance and root development. Before discussing the mechanism of this effect it is of interest to point out that there is evidence that the control of root development by carbohydrate-nitrogen balance in Lemna colonies is a particular case of a general phenomenon. Chandler (1919), Gericke (1922), Turner (1922), Crist and Stout (1929), and others have associated relatively marked development of roots with a deficiency of nitrogen in such a diversity of plants as cereals, fruit-trees, and vegetables. Muenscher (1922), Shirley (1929), Reid (1929), and others have shown that a high level of the light factor favours increased development of roots relative to that of shoots in cereals, conifers, and seedlings of herbaceous plants. Increase of assimilation by enrichment of the atmosphere with CO_2 increases the growth of roots more than that of shoots in cereals and the Leguminosae (Reid, 1929*a*), whereas decrease of assimilation by defoliation and pruning reduces subsequent root growth to a relatively greater extent than shoot growth in fruit-trees (Chandler, 1919), grasses (Parker and Sampson, 1930), and the tomato (Kraus and Kraybill, 1918). Moreover, Reid (1926) concluded that the ratio of shoots to roots in germinating seedlings of nineteen different plants is related to the chemical composition of the seed, a high ratio of shoots to roots being associated with high nitrogen content.

Observations from a number of experiments have shown that the length of root of Lemna plants is a highly sensitive indication of carbohydrate-nitrogen conditions within the frond. In one case reduction in nitrate content of the solution caused an increase in mean length of root of the colony of over 100 per cent. in four days, whereas the corresponding changes in mean frond area and dry weight over the same period were of the order of 5 per cent. and could not be considered significant. In another experiment colonies were grown in a series of culture solutions of increasing nitrate content. In solutions of nitrogen level greater than 5 mg. per litre no differences could be detected in rate of multiplication or net assimilation, but the length of root was significantly decreasing as the nitrate supply was increasing, and this decrease was associated with paler frond colour and presumably decreasing chlorophyll content.

Mason and Maskell (1928) have shown that the direction of travel of carbohydrates in the cotton plant is determined by diffusion gradients. If it is assumed that a diffusion gradient determines the supply of carbohydrates to the developing root-meristem of Lemna plants a simple mechanism is suggested for the dependence of relative root development on a carbohydrate-nitrogen balance. In the frond meristem and the root meristem carbohydrates and nitrogenous compounds are required in balanced concentrations corresponding to those of utilization in development. Any excess of carbohydrate above that required for development of the frond meristem raises the concentration in the frond and so steepens the diffusion gradient to the developing root meristem. Reduction in intensity of light and corresponding rate of carbohydrate synthesis would lead to a lower carbohydrate concentration in the fronds and a less steep diffusion gradient to the root. Increase in nitrogen supply unaccompanied by corresponding increase in rate of carbohydrate synthesis would lead to the same situation, for the increase in rate of development of frond meristem due to increased nitrogen supply would be associated with increased carbohydrate utilization in the frond and a reduction in the carbohydrates available for transport to the root. Such a mechanism would tend to an automatic adjustment of the balance of the plant, for a longer root, resulting from excess of carbohydrate over nitrogen, should absorb more nitrate, and a shorter root, resulting from excess of nitrogen over carbohydrates, should absorb less.

SUMMARY

Length of root is a sensitive indicator of the carbohydrate-nitrogen balance in the fronds of Lemna. Under sixteen combinations of light intensity and nitrogen supply the development of the root is significantly correlated with the ratio between starch and protein, as estimated by the intensity of colour obtained by staining whole plants with iodine and Millon's reagent. The mechanism of the relation between root development and carbohydrate-nitrogen balance is discussed.

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A Contribution to the Study of the Nuclei of Peziza rutilans Fries

BY

IRENE M. WILSON, M.Sc.

(*Department of Botany, University College of Wales, Aberystwyth*)

With Plates XXIV and XXV and ten Figures in the Text

INTRODUCTION

THE cytology of the ascus of *Peziza rutilans*[1] was first described by Guilliermond in a series of papers published in 1904 and 1905. In these it was established that a numerical reduction in the chromosome number occurred during the first division of the nucleus in the ascus. The number of bivalent chromosomes in the prophase of the first division was sixteen, and sixteen chromosomes were present throughout the second and third mitoses in the ascus.

In 1907 and 1908 a very full account of the cytology of *Humaria rutilans* was given by Fraser. She found that sexual organs were not formed, but that apogamous fusions of pairs of nuclei occurred in the hypothecium, and concluded that the cells containing fusion nuclei gave rise to ascogenous hyphae. Sixteen chromosomes were present in the divisions in the ascogenous hyphae. A second nuclear fusion occurred in the penultimate cell of the ascogenous hypha. The first two divisions in the ascus were respectively heterotype and homotype, showing the stages described by Farmer and Moore (1905) for the reducing divisions of animals and plants. Sixteen gemini were present in the prophase of the first division and, after transverse fission on the spindle, sixteen chromosomes, V-shaped on account of a longitudinal split, passed to each pole in the anaphase. Sixteen chromosomes reappeared in the second division and sixteen, straight or bent according to the position in which they were attached to the spindle, passed to opposite poles in the anaphase. Of the sixteen curved chromosomes present in the prophase of the third division, eight were held to pass bodily to each pole of the spindle. Thus two reductions were described in the chromosome number in the ascus, the first compensating

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[1] Although Guilliermond named his fungus *Peziza rutilans* Cooke, and expressed some doubt as to its identity with the species of Fries and Quélet, Fraser (1908) concluded that his species was the same as that described by her as *Humaria rutilans* Fries. This interpretation, which appears to have been accepted by Guilliermond, has been followed in the present paper. I am indebted to the authorities of the British Museum (Natural History) for naming the fungus which is the subject of this study and for the information that it is the same species as that investigated by Fraser.

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the apogamous fusion in the hypothecium, and the second the fusion in the ascogenous hypha. To the second reduction, the name brachymeiosis was given.

After a re-examination of his slides, Guilliermond (1911) expressed himself in substantial agreement with Fraser regarding the first two divisions, but held that there was no reduction in the third division. He considered that the number of chromosomes at the telophase of this division was greater than eight and approaching sixteen, although it was impossible to count them exactly owing to their length and twisting.

I am extremely grateful to Professor Dame Helen Gwynne-Vaughan for her suggestion that it might now be worth while to reinvestigate this fungus, making use of any advances in technique, and for allowing me to examine the preparations from which the figures for her paper (Fraser, 1908) were drawn. It seems unnecessary to add that I can confirm the accuracy of the representation of all the preparations which I have examined. The nuclei of *Peziza rutilans* are peculiarly suitable for detailed study owing to their exceptionally large size compared with those of other fungi.

Material and Methods

A supply of *Peziza rutilans* was obtained from Smith's Lawn, Windsor Great Park. It was growing on sandy soil, at the side of a path and on a rubbish dump, and was frequently but not invariably associated with species of Polytrichum and Cladonia. Ascocarps were available from the middle of September until the beginning of December each year.

All the material was fixed under an air pump in the field. Several fixatives were tried, the most useful being Flemming's strong fluid diluted with an equal volume of water (Gwynne-Vaughan and Barnes, 1930). The modifications of Flemming's fluid known as 2 BE and 2 BD also gave satisfactory results (La-Cour, 1931). Bouin's fluid (Chamberlain, 1933) caused some swelling, especially of the cell-walls. After washing for about six hours in running water, in the case of the Flemming mixtures, and taking up through the alcohols, the ascocarps were examined under a dissecting microscope and any adhering sand grains were removed. They were embedded through chloroform in paraffin wax of m.p. 52° C. Sections were cut from 5–20 μ, but chiefly at 12 or 15 μ. An alcoholic solution of chlorine was employed for bleaching after fixatives containing osmic acid.

Some sections were stained in Heidenhain's iron-alum-haematoxylin counterstained with erythrosin in clove oil. This was particularly good for the early stages of the first division in the ascus and for the cytoplasm and spindle at all times. La-Cour's modification of Newton's method for gentian violet (La-Cour, 1931) was, however, found to give much clearer preparations and was essential in elucidating critical anaphase stages. When a counterstain was required for the gentian violet, light green or orange G in clove oil was used.

For the Feulgen reaction, using material fixed in Flemming's fluid half strength, a period of hydrolysis of eighteen minutes in normal hydrochloric acid followed by one hour in fuchsin sulphurous acid gave good results (Feulgen and Imhäuser, 1925; Bauer, 1932; Gardiner, 1935).

The mature ascocarp.

The mature ascocarp has a hypothecium of loosely woven hyphae bounded by a more compact pseudoparenchymatous rind, from which grow out numerous, branched, septate hyphae. These have a nutritive function since they are frequently seen in contact with decaying plant remains and are often filled with dense granular contents. Their nuclei may be rounded, or more or less elongated (Text-fig. 2).

Within the rind of the ascocarp, small pieces of decaying plants, old pollen grains of Pinus, and even small colonial algae such as Kirchneriella are sometimes included.

In the looser central tissue, the majority of the cells are small with scanty contents and one or a few rounded nuclei. Conspicuous in this spongy framework are hyphae taking an unusually straight course with septa at infrequent intervals and long spindle-shaped nuclei pressed in single file against their walls (Text-fig. 1). So peculiar did these nuclei appear, that some doubt was felt as to their nature until the Feulgen reaction was carried out, when the chromatin threads were brightly coloured. The elongation of the nuclei does not appear to be caused by the narrowness of the hypha as is the case in the paraphyses (Text-fig. 4). These hyphae can be traced up from the base of the ascocarp, through the hypothecium branching at first occasionally, then more profusely in the sub-hymenial region and into the paraphyses. They are probably concerned in the translocation of food materials.

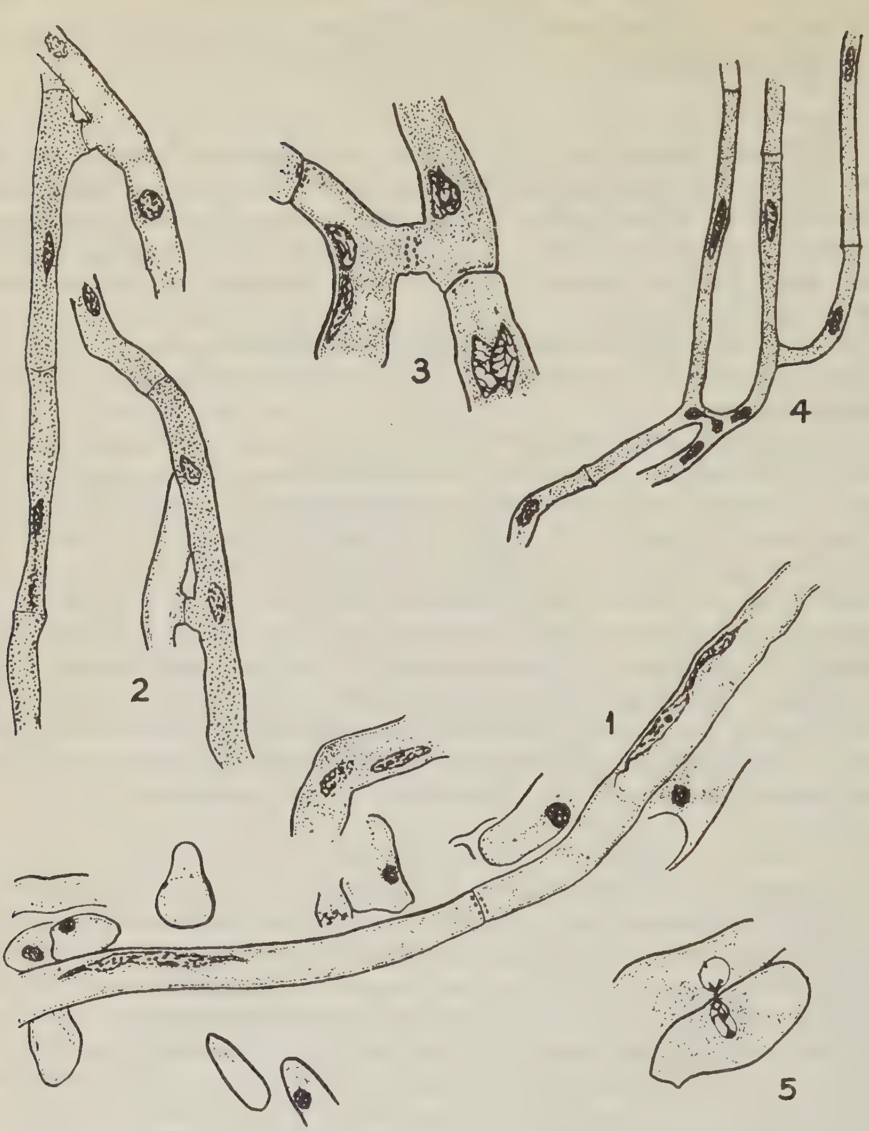
HYPHAL FUSIONS

Hyphal fusions have been seen in almost all the vegetative parts of the ascocarp. They occur in the hyphae growing out round the bottom of the ascocarp (Text-fig. 2), in the compact pseudo-parenchymatous rind at the base (Text-fig. 3), and, in old ascocarps, among the hyphae which form the paraphyses (Text-fig. 4) in the sub-hymenial and hymenial regions. All such fusions are of the 'peg-to-peg' type (Buller, 1933). Occasionally a single cell has been seen connected at the same time with two other hyphae. An open pore is made in the connecting bridge, leaving a free passage not only for food materials but also for nuclei (Text-fig. 4).

The arrangement of the nuclei shown in Text-fig. 3 is probably without significance. Hyphae with nuclei in pairs have been seen at all levels in the hypothecium, but, as far as can be ascertained, they have no connexion with the ascogenous hyphae.

MIGRATION OF VEGETATIVE NUCLEI

On three occasions a nucleus has actually been seen in the bridge formed by a 'peg-to-peg' fusion, all three cases being in the paraphyses (Text-fig. 4).



TEXT-FIGS. 1–5. Fig. 1. Hypha with long nuclei in hypothecium. × 1,000. Figs. 2–4. Peg-to-peg hyphal fusions. Fig. 2. Hyphae growing from base of ascocarp. × 1,000. Fig. 3. Base of ascocarp. × 1,000. Fig. 4. Base of paraphyses. Note nuclear migration across bridge. × 1,000. Fig. 5. Nuclear migration in sub-hymenium. × 1,500.

Nuclear migrations having no connexion with hyphal fusions have also been seen occasionally, in one instance at the extreme base of an ascocarp, and in two cases in the sub-hymenium. Only in one of the latter was it clear that the migration occurred between two cells of different hyphae. In any event, a very small pore is formed, so that the nucleus is deeply constricted, assuming the form of a figure 8, when half-way through the opening (Text-fig. 5).

THE QUESTION OF FUSION OF VEGETATIVE NUCLEI

No decisive evidence has been obtained of the fusion of vegetative nuclei in pairs as found in the material studied by Fraser (1908). Nuclei have, however, sometimes been seen lying in very close contact, and it is in some cases extremely difficult to say with certainty whether their nuclear membranes are still intact. Such paired nuclei are found, not only in the sub-hymenium, but also at the extreme base of the ascocarp and in the sheath. If these be fusions, the diversity in their distribution and even more, the cytological evidence, do not permit the conclusion that cells containing fusion nuclei initiate the ascogenous hyphae.

Occasionally very large cells have been seen in old ascocarps which are renewing vigorous growth after a period of inactivity and also deep down in the hypothecium of a young ascocarp. Such cells occur only sporadically and do not appear to give rise directly either to paraphyses or to ascogenous hyphae, although growth is very prolific in their neighbourhood. They contain dense, vacuolate cytoplasm and many large rounded nuclei. They are comparable in some respects with the 'storage cells' containing fusion nuclei, described in *Leotia lubrica* and *Lachnea scutellata* (Brown, 1910, 1911) and *Lachnea stercorea* (Raymond, 1934). In appearance, however, the large cell of *Peziza rutilans* more nearly resembles that believed to represent a 'disused ascogonium' in *Otidea aurantia* Mass. (Fraser and Welsford, 1908).

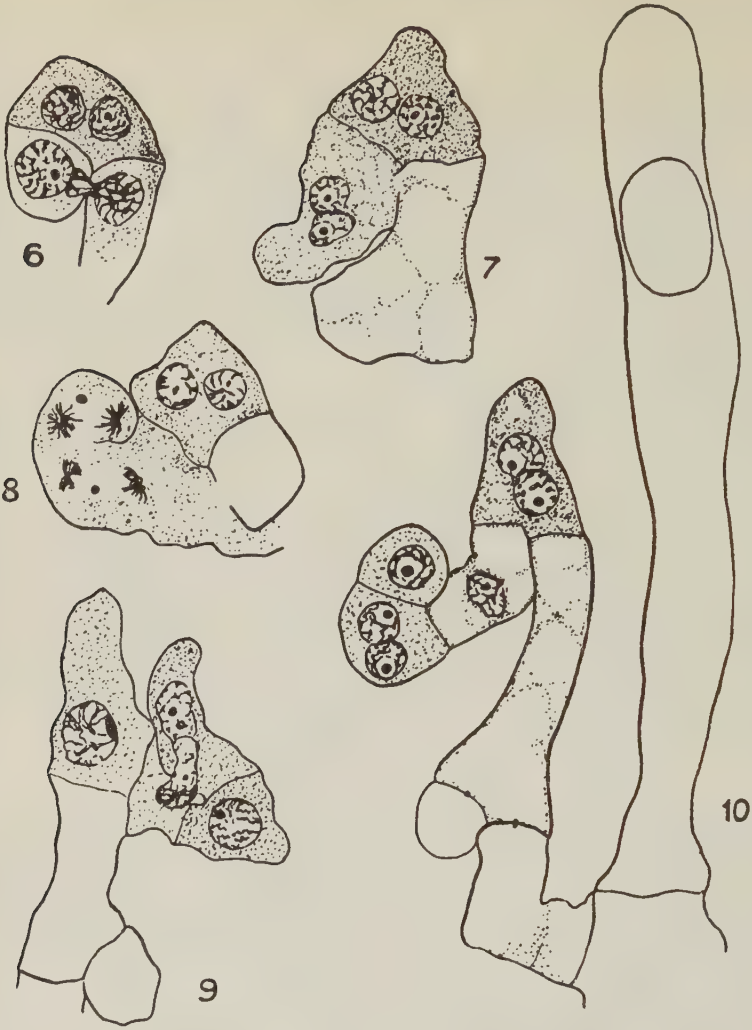
ASCOGENOUS HYPHAE

Ascogenous hyphae are conspicuous in the sub-hymenium, but it has not been possible to trace their origin. It is probable that at least one mitosis occurs, affecting all the nuclei in the hypha, before the tip bends over to form a crozier. In the lower parts of the hypha the nuclei may be crowded after a recent division, but in the upper parts they are in single file.

Either before or after the hook is formed, the two nuclei nearest the tip undergo a simultaneous mitosis (Pl. XXIV, Figs. 1, 2) and walls are formed cutting off a uninucleate end cell, a binucleate cell next to it, and a stalk cell. The penultimate cell may form another crozier (Text-figs. 9, 10) with the simultaneous mitosis of its two nuclei, or the nuclei may fuse and the cell become an ascus (Pl. XXIV, Figs. 3, 4).

PROLIFERATION OF ASCOGENOUS HYPHA

Sometimes even before the two nuclei in the penultimate cell of the crozier have fused or entered on a mitosis, the uninucleate terminal cell and stalk cell come into contact and an open pore is formed between them. This process, which the writer has watched in living material of *Sepultaria sepulta*, takes place rapidly, occupying less than ten minutes. A nucleus then passes from the stalk cell to the terminal cell, or more often, from the terminal cell



Text-figs. 6–10. Proliferation of ascogenous hypha. Fig. 6. Migration of nucleus from stalk to terminal cell. × 1,500. Fig. 7. Two nuclei in terminal cell. × 1,500. Fig. 8. A nucleus has migrated from terminal cell to stalk and the two nuclei of the stalk are undergoing a simultaneous mitosis for the new crozier. × 1,500. Fig. 9. Three croziers have been formed, the second and third of which will form asci. Nuclear migration in progress for fourth. × 1,500. Fig. 10. Four successive croziers. Last is upside down. × 1,500.

to the stalk cell (Text-figs. 6–10). The binucleate cell thus formed grows out into another crozier after its two nuclei have undergone a simultaneous mitosis (Text-fig. 8). The same process may be repeated in the new crozier, so that a large number of asci may result from the proliferation of the original crozier (Text-figs. 9, 10). The proliferation of the crozier was noted by

Fraser (1908) for *H. rutilans*, and is now a well-known phenomenon among the Ascomycetes.

In discussing Claussen's work (1912) on the ascogenous hyphae of*Pyronema confluens*, Martens (1932) points out that each crozier gives only one ascus for certain, but if the stalk and the tip of the crozier always fuse, two asci are possible from each crozier. The fact is, however, that the process need not stop there; it may be repeated indefinitely, so that theoretically the original crozier may give rise to an unlimited number of asci.

As proliferation proceeds, it is seen that only the growing tip of the ascogenous hypha contains living contents, the older parts are left almost empty (Text-figs. 9, 10). Although an open pore may be seen in the basal wall of a young ascus, the old emptied cells seem to have their walls plugged.

It is largely because the lower parts of the ascogenous hyphae have lost most of their contents that they are so difficult to trace back to their source.

Evidences of proliferation have been seen in every ascocarp in which it has been looked for, and it does not occur only occasionally, but in great abundance. The conclusion seems to be inevitable that the majority of asci in a mature ascocarp have been formed in this way.

Even in a stage where only uninucleate asci are present, there are indications that two or three croziers may have been superimposed before an ascus is formed. It is an interesting theoretical consideration that if the ascogenous hypha behaved in this way from its inception, each of the two nuclei in the penultimate cell would be descendants of the two original nuclei. This is not so, however, in *Peziza rutilans*.

It has frequently been noticed that, in a single ascocarp, one particular stage of proliferation has been reached in a great many different parts of the ascocarp at the same moment. In the hymenium, also, many asci have simultaneously attained the same stage in division. These successive crops of asci have been produced by the successive croziers formed by the proliferating ascogenous hyphae.

This process maintains a 'mixed' condition of the hymenium, with asci at various stages of development. The lateral expansion of the hymenium is ensured, while the occasional production of a crozier instead of an ascus by the penultimate cell raises the level of the hymenium as the ascocarp matures and the cup opens more widely.

Text-figs. 9 and 10 show that, after a while, a crozier may be formed upside down. This readily accounts for the asci which can often be seen growing down through the hypothecium in old fruit bodies. Such asci may often contain mature ascospores, though they have also been seen with nuclei degenerating at an earlier stage.

MITOSIS IN THE CROZIER

In the prophase and metaphase of the simultaneous mitosis in the crozier there are sixteen V-shaped chromosomes in each nucleus (Pl. XXIV, Fig. 1).

These split longitudinally and sixteen V-shaped chromosomes pass to each pole (Pl. XXIV, Fig. 2). The spindle is intranuclear and has a well marked crescent-shaped centrosome at each end. The four daughter nuclei then enter a resting stage which may be of short duration.

Of the four nuclei in the crozier, the two in the penultimate cell are sisters of those in the stalk and distal cell respectively. The two in the penultimate cell usually fuse to form the definitive nucleus of the ascus, which then undergoes meiosis, while the other two (after one has migrated) pass through an ordinary mitosis. Theoretically, then, it is probable that the premeiotic division does not differ from that before a somatic mitosis, but the nuclei are too small to ascertain exactly the time at which the longitudinal split occurs.

Fusion in the Ascus

Whether the reticulate appearance of the interphase nucleus be an artefact or not, it is at least a characteristic one after certain types of treatment and makes this stage easily recognizable (Sharp, 1929, 1934). By this criterion, when the migration of a nucleus between the stalk and distal cell of the crozier occurs, the nuclei are in a resting condition. In some cases, however, the anastomoses are very fine, and certain strands, probably representing the prophase chromosomes of the next division, stand out very distinctly (Pl. XXIV, Fig. 4). The interphase is of short duration and it is indeed difficult to draw a sharp line between it and the early stages of the prophase.

Similarly, the nuclei which fuse in the penultimate cell of the crozier to form the definitive nucleus of the ascus show a reticulum or else certain strands are well marked, so that perhaps the prophase is just beginning (Pl. XXIV, Fig. 3).

The two nuclei simply merge into each other. The fusion nucleus shows a reticulum with a fine mesh suggesting a metabolic stage (Sharp, 1929) (Pl. XXIV, Fig. 4). The two nucleoli at first lie freely within the encompassing spherical network. Then they fuse.

First Division in the Ascus

The chromatin thread is soon seen to consist of separate chromosomes showing conspicuous coils (Pl. XXIV, Fig. 5). These are probably of the type described by Darlington (1937) as the relic spirals. One after another they straighten out and become gradually spread through the nuclear area. As far as can be ascertained the stretched thread is single at this stage. It appears to be inconspicuously beaded owing either to the presence of chromomeres, or, more likely, to the remains of the spiral. This is what used to be described as the continuous spireme stage, but in some preparations it is clear that the chromosomes are separate bodies here as in earlier and later stages. Pl. XXIV, Fig. 6, shows pairing in progress with some single and some double strands. After the chromosomes have paired they remain very closely associated (Pl. XXIV, Figs. 7, 8) and slightly rolled round each other.

In this and the earlier zygotene condition, collapse of the chromosomes to one side of the nucleus is very liable to occur.

While the ascus is quite short, opening out between the chiasmata of the paired chromosomes begins, giving the diplotene condition. The nucleus remains in this stage while the ascus grows up and reaches nearly its full stature just below the level of the tops of the paraphyses. In late diplonema it is possible to see that the loops between successive chiasmata lie in different planes and that the separating threads are double (Pl. XXIV, Fig. 9). It is probable that the division of the chromosomes into two chromatids occurred earlier than this.

The number of chiasmata is difficult to determine with certainty because there may still be a certain amount of twisting of the looped threads. It is, however, fairly high, averaging about five to eight for each pair (Pl. XXIV, Fig. 9). In a number of cases there is no distal chiasma, so that the ends of the paired chromosomes diverge freely.

Up to this point there are always some chromosomes in contact with the nucleolus.

The structure of the nucleolus itself shows some interesting features. During the early prophase, it is situated inside the nucleus and is large, more or less spherical, and always shows a few brownish, highly refractive granules in its interior. As diplonema progresses, it becomes pressed against one of the ends of the much enlarged ovoid nucleus and becomes highly vacuolate.

As diakinesis approaches, the bivalent chromosomes shorten (Pl. XXIV, Fig. 10) and are spread round the periphery of the nucleus. The chiasmata seem to be reduced in number, but are not completely terminalized. One loop is now seen to be conspicuously inflated compared with the rest, and it stains less deeply with haematoxylin. It marks the position of the future double attachment to the spindle. It always occupies a median or sub-median position (Pl. XXIV, Fig. 11).

The double nature of the separating loops of the chromosomes can still be seen occasionally, but is gradually obliterated.

The development of the spindle of this fungus has already been fully described (Guilliermond, 1905; Fraser, 1908). The centrosome at the periphery of the nucleus divides and the two daughter centrosomes move apart, each with its cone of spindle fibres, until they reach opposite poles of the nucleus. The fully developed spindle has well-marked, crescent-shaped centrosomes and fairly conspicuous asters. The gemini remain near the periphery of the nucleus during the development of the spindle and appear to be passively drawn on to it.

At metaphase (Pl. XXIV, Fig. 12) the gemini are very crowded on the spindle, and some have to lie above or below the level of the equator. Each bivalent chromosome lies with its two centromeres stretched apart on the long axis of the spindle. The most common form of the bivalent at this stage is either a simple diamond shape indicating complete terminalization with median attachment, or more frequently, a diamond shape with one or two loops on one or both of the distal arms of the paired chromosomes, indicating incomplete terminalization.

The anaphase separation of the chromosomes sets in gradually. Pl. XXIV, Fig. 12, shows most of the bivalents in the full metaphase position, while two pairs have started to move apart. Small pairs with complete terminalization are probably the ones which divide first. As they separate the double nature, seen earlier in the diplonema, may once again become apparent (Pl. XXIV, Fig. 13).

In anaphase the chromatids may diverge widely, giving the appearance of two V's united at the base, or, if the ends of the chromatids come into contact, like two O's leaning away from each other. In other cases, divergence may only occur in one limb, or the chromatids may remain closely associated in both arms so that a simple, rather thick V results. Most of these forms of anaphase chromosomes may be seen in Pl. XXIV, Fig. 15.

Sometimes there is a marked lag in the pulling apart of the chromosomes at anaphase. Pl. XXIV, Fig. 14, shows two such pairs from different nuclei. They are still on the equator of the spindle while all the other chromosomes are well on their way to the poles, and it seems probable that chiasmata present an obstacle to separation.

If the attachment is not quite median, the shorter arm of the chromosome breaks away from its partner before the other and becomes much contracted, so that the whole chromosome presents temporarily the form of a J. When both limbs have broken apart, both are contracted and the chromosome is a short thick V or some modification of this caused by the divergence of its constituent chromatids, as already described.

Those which have only just separated, and are still near the equator of the spindle, often have one of the two chromatids drawn out into a long thin strand, suggesting that they were pulled out by the breaking of a chiasma, and that this affected only one chromatid of each pair (Pl. XXIV, Fig. 15).

When all the chromosomes have separated, the spindle elongates markedly, so that the two groups of daughter chromosomes are moved far apart. The chromatids remain visible as separate strands throughout the short interphase between the first and second divisions (Pl. XXIV, Fig. 17). They look like long, thin threads. Their granular appearance is probably due to the spiral structure of the chromonema, which is more plainly visible in the very early prophases of the second division.

The nuclear membrane disappears at metaphase and the nucleolus is extruded into the cytoplasm. It may remain visible for some time, lying between the two daughter nuclei, even after they have formed their own nucleoli.

The number of bivalents can be counted most easily at diakinesis, but it is also possible to do so at diplonema and at metaphase. There are sixteen paired chromosomes present at all these stages and sixteen pass to each pole at anaphase (Pl. XXIV, Figs. 9, 10, 11, 12, 13, 15). If sections are cut too thin, so that the nucleus is disturbed, the chromosomes may be broken. This is particularly liable to occur at two stages, diakinesis and anaphase. In the former, fragments are recognizable by their small size and sometimes by the absence of the attachment region, while in the latter stage a high proportion of thin single V's is significant.

SECOND DIVISION IN THE ASCUS

In the prophase of the second division, the same shapes which characterized the anaphase of the first division reappear. Now, however, the chromosomes are longer and thinner, and their chromatids diverge widely or may be wrapped round one another so that quite complicated looking forms result (Pl. XXIV, Figs. 18, 19 *a*, *b*).

The spindle arises as in the first division. The sixteen longitudinally split chromosomes are arranged somewhat irregularly on it (Pl. XXIV, Fig. 20). The divergent arms of the chromatids then close and separation of the daughter chromosomes begins at the region of the centromere (Pl. XXIV, Fig. 19 *c*, *d*). Sixteen more or less V-shaped chromosomes pass to each pole of the spindle (Pl. XXIV, Fig. 21).

The nucleolus is extruded and the daughter nuclei are separated by the elongation of the spindle as in the first division. The long axis of the spindle in both the first and second divisions lies parallel with the long axis of the ascus, or nearly so.

The first two divisions in the ascus constitute a meiotic phase. There are sixteen bivalents in the prophase of the first division and sixteen chromosomes in the second division. The anaphase chromosomes are typically V-shaped in both divisions as in the mitosis in the crozier.

THIRD DIVISION IN THE ASCUS

There may be a definite resting stage between the second and third division in the ascus, during which the four nuclei increase considerably in size. The chromatin has the form of a network, but often with some zig-zag strands more clearly marked than the connecting anastomoses, representing the telophase chromosomes of the last division and the prophase chromosomes of the next (Pl. XXV, Fig. 22). This appearance has been seen to be characteristic of the resting nuclei of the crozier (Pl. XXIV, Fig. 4). As prophase sets in the chromosomes become distinct as separate entities and each consists of two chromonemata which are spirally coiled (Pl. XXV, Fig. 23). The V-shaped chromosomes of late prophase (Pl. XXV, Fig. 24) still show two chromatids, but they are no longer coiled themselves, though they may be twisted round each other. This relational coiling and the longitudinal split are obliterated as the chromosomes shorten and thicken for the metaphase (Pl. XXV, Fig. 25).

There is a marked polarity in the arrangement of the chromosomes during the prophase of the third division. As in the telophase of the previous division (Pl. XXV, Fig. 24), the apices of the sixteen V-shaped chromosomes form a small circle from which their long, divergent limbs extend. The apices are too crowded to lie exactly in one plane.

The spindles arise as in the first and second divisions. They usually lie at right angles to the long axis of the ascus, but this is by no means always the case. Some may lie longitudinally and others horizontally in the same ascus, and they may even cross each other. The spindle has crescent-shaped centrosomes and very well-marked asters.

It is difficult to demonstrate the spindle and to count the number of chromosomes present in the same nucleus, partly because the spindle is best seen in side view, while the chromosomes are most easily counted from a polar view, but also because they require different degrees of differentiation in staining. Fortunately, in a few asci the spindle is visible in some of the nuclei, while others in the same ascus lie in such a way that a count can be made of the chromosomes.

In this way it has been possible to make sure that sixteen V-shaped chromosomes are present on the spindle. They do not lie in pairs, and it would be mechanically impossible for them to pair after they are on the spindle.

Pl. XXV, Fig. 25, shows most of the chromosomes at full metaphase with no longitudinal split visible, but in two of these chromosomes, drawn separately in Pl. XXV, Fig. 26, the split seen in the prophase is again apparent and separation has begun in the region of the spindle attachment. A similar spread of the onset of anaphase, the chromosomes being affected one after the other, was seen in the first and second divisions.

At later stages in the anaphase, shapes characteristic of the separation of two V's can be seen. Since the chromosomes have not paired, these must be due to the separation of the two chromatids of a longitudinally split V-shaped chromosome. These are drawn separately for a single dividing nucleus in Pl. XXV, Fig. 28, while the whole nucleus is represented in Pl. XXV, Fig. 27. Similar shapes can be seen in Pl. XXV, Fig. 29, where, however, only part of the nucleus has been drawn.

The diamond shapes seen so frequently in full anaphase (Pl. XXV, Figs. 27, 28, 29) are interpreted as two V-shaped daughter chromosomes with their centromeres separated, but with the tips of the limbs still attached. The W-shapes similarly show two V-shaped daughter chromosomes with the attachment regions more or less separated, but with only one limb of each united to its partner. In the double J's the freed limbs may have contracted considerably and the centromere may also be sub-median. When fully separated both limbs are very much shortened.

Contraction occurs only after the breaking apart of the limbs of the V-shaped daughter chromosomes, and at late anaphase (Pl. XXV, Fig. 30) and at telophase (Pl. XXV, Fig. 31) sixteen fairly small V's can be counted.

It will be noticed that in the anaphase (Pl. XXV, Figs. 27, 29) the chromosomes are very long and the crowding on the spindle is so acute that only some lie on the equator, the rest are distributed over the spindle, often lying quite close to one of the poles. In spite of this irregular distribution, the division takes place normally.

It is so difficult to make an exact count of the chromosomes at the anaphase of the third division, owing to their large size and crowded arrangement, that this has only been done completely once. In this case sixteen chromosomes in various stages of division were found (Pl. XXV, Figs. 27, 28). Counts of more than twelve have, however, been made several times. In every third anaphase seen, diamond and W-shapes, similar to those figured and held to be characteristic of a dividing V-shaped chromosome, have been seen. Camera lucida drawings made to compare the size of the nucleus at this stage in different asci show no appreciable variation in size. For these reasons it is concluded that the one case fully worked out represents a normal state of affairs.

It is somewhat easier to make counts when the V-shaped chromosomes have just reached the poles. Sixteen have been counted in thirteen different asci belonging to several ascocarps, while it has been possible to count numbers approaching sixteen (i.e. more than twelve) in many more.

At full telophase the individual V's may be rather obscured, but the number of ends visible should theoretically be thirty-two. Actually varying numbers have been counted ranging from sixteen to twenty-seven. It is not considered that the low number of sixteen can be due to the separation of eight whole V-shaped chromosomes to each pole, partly because the number of ends is not sixteen *or* thirty-two but varies widely between these figures, but principally because of the constancy of the appearance of dividing chromosomes and the high numbers always present in the anaphase. Moreover, an exactly similar variation has been found in the number of ends seen in the telophase of the division in the crozier and of the second division in the ascus where there is no doubt that sixteen V-shaped chromosomes are present. The low numbers may perhaps be accounted for by the closeness of the chromosomes at this stage, so that either adjacent limbs easily collapse on to each other in fixation, or if the preparation is over-stained they are not differentiated. A count of the number of apices of the V-shaped chromosomes (Pl. XXV, Fig. 31) is more significant than the number of ends and may show sixteen spaced evenly apart, although the limbs of adjacent chromosomes lie in contact.

SPORE FORMATION

Spore formation has already been fully described for *Peziza rutilans* (Fraser, 1908) and it appears to follow the lines common among other Ascomycetes. The ring formed by the apices of the V-shaped chromosomes of the third telophase is pushed up as a cylindrical projection, but later it becomes long and pointed and it looks as if the astral rays bent round from its apex and delimited at least part of the inner wall of the spore (Pl. XXV, Fig. 32).

In two asci (Pl. XXV, Figs. 32, 33), spores have been found containing no nucleus, though in one case a colourless, refractive spot was present. Lindegren (1934) has described akaryotic spores in Neurospora. Four were present in an abnormal ascus of *N. crassa*, and large numbers were obtained by inbreeding certain pure-bred stocks. His conclusion, that perhaps 'the mechanism governing the production of spore walls enjoys a certain amount of autonomy', is supported by the observations in *P. rutilans.*

The spores are normally uninucleate, but asci containing abnormal spores, with two, three, four, or five nuclei, are by no means uncommon. Pl. XXV, Fig. 33, showing an akaryotic spore, also shows one with three nuclei. Multinucleate spores were described by Fraser (1908) in this species.

MITOSIS IN VEGETATIVE NUCLEI

It was hoped to obtain confirmation of the haploid number of chromosomes by counts in the first division of the spore, but all attempts to induce germination have so far failed. Many factors which might influence germination, such as age, temperature, action of stimulants, nature and pH of the medium, mechanical breaking of the spore coat, and even digestion by a beetle which was found eating old ascocarps of the fungus, have been tried, but the right combination of conditions has not yet been found.

Dividing nuclei have frequently been seen in the paraphyses. No reliable count is possible here, however, because the nucleus is very much compressed by the narrowness of the hypha and the chromosomes are long and very much entangled.

Quite a number of nuclear divisions occur in the cells at the base of a mature ascocarp and in the sheath. Those in the sub-hymenium are not always easy to distinguish with certainty from ascogenous hyphae, and they have therefore been disregarded.

The dividing vegetative nuclei are large compared with those in a resting stage and approach in size those at a corresponding stage in the third division.

The prophase chromosomes are V-shaped, as in the other divisions, and about sixteen are present (Pl. XXV, Fig. 34). Anaphase stages show the shapes familiar in other divisions and characteristic of a dividing V, but no counts have been made at this stage. At telophase only about sixteen ends have been counted, as was sometimes the case in the crozier, and in the second and third divisions in the ascus.

The chromosome counts during mitosis in vegetative nuclei therefore, so far as they go, confirm the findings in the ascus divisions, but they cannot be regarded as conclusive in themselves.

Since both the sexual fusion and the second reduction are lacking, it is evident that the fungus dealt with in the present investigation is completely apogamous.

SUMMARY

1. Hyphal fusions occur in all vegetative parts of the ascocarp.

2. Migration of nuclei has been observed in the extreme base of the ascocarp, in the sub-hymenium, and in the paraphyses.

3. The ascogenous hypha bends over to form a crozier in the usual way, and the ascus develops from the binucleate penultimate cell after fusion of its two nuclei.

4. Proliferation of the ascogenous hyphae occurs, giving successive crops of asci.

5. There are sixteen V-shaped chromosomes in the nuclei undergoing a simultaneous mitosis in the crozier.

6. The first two divisions in the ascus constitute a meiotic phase. Sixteen bivalents are present in the first division. They have median or sub-median attachment to the spindle. At anaphase sixteen more or less V-shaped chromosomes, often double on account of a longitudinal split, pass to each pole. In the second division sixteen similar chromosomes reappear and in the anaphase the split seen in the first division takes effect, so that sixteen single V's pass to each pole.

7. The third division is an ordinary mitosis. The chromosomes are longitudinally split in the prophase. Sixteen more or less V-shaped chromosomes are present in metaphase and sixteen V-shaped daughter chromosomes pass to each pole in the anaphase.

8. There are also sixteen V-shaped chromosomes in the prophase and metaphase of vegetative nuclei dividing at the extreme base of the ascocarp.

9. It is therefore concluded that in the material investigated there is only one nuclear fusion, namely, that in the penultimate cell of the crozier, and the compensating reduction in the chromosome number occurs in the first division in the ascus.

The earlier part of this work was carried out in the Botanical Department at Birkbeck College, University of London, under the direction of Professor Dame Helen Gwynne-Vaughan, to whose unfailingly helpful advice and criticism throughout I am greatly indebted. I also gratefully acknowledge the generous assistance received from the Dixon Fund of the University of London, which later enabled me to obtain suitable instruments to continue this research.

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EXPLANATION OF PLATES XXIV AND XXV

Illustrating Miss Irene M. Wilson's paper on 'A Contribution to the Study of the Nuclei of *Peziza rutilans* Fries'

PLATE XXIV

Fig. 1. Mitosis in crozier showing sixteen V-shaped chromosomes. × 2,250.

Fig. 2. Anaphase of mitosis in crozier. × 2,250.

Fig. 3. Fusion of two nuclei in penultimate cell of crozier. Two nucleoli distinct. Reticulum present. × 3,000.

Fig. 4. Fusion complete in penultimate cell of crozier. Nuclei in interphase. × 3,000.

Fig. 5. Prophase of first division in ascus. Separate chromosomes, some showing spiral, others a slightly beaded appearance. × 3,000.

Fig. 6. Prophase of first division, showing (*a*) single thread with relic spiral and (*b*) double thread. × 3,000.

Fig. 7. Prophase of first division. Double nature of thread seen in places. × 3,000.

Fig. 8. Single-paired chromosome from a nucleus at the same stage as in Fig. 7. × 3,000.

Fig. 9. First division in ascus. Diplotene stage. The separating loops are double (*a*). × 3,000.

Fig. 10. First division in ascus. Diakinesis. Sixteen bivalents. Nucleolus vacuolate and irregular. × 3,000.

Fig. 11. First division in ascus. Curved spindle present. Inflated attachment region conspicuous in several bivalents. × 3,000.

Fig. 12. First division in ascus. Most bivalents in full metaphase position, but anaphase separation has begun in two small pairs (*a* and *b*). × 3,000.

Fig. 13. A slightly later stage than Fig. 12. Chromosomes drawn separately but relative position on spindle shown. Longitudinal split apparent in limbs of some daughter chromosomes after separation. × 3,000.

Fig. 14. First division in ascus. Two bivalents from different nuclei which show 'lag' in anaphase separation. × 3,000.

Fig. 15. Anaphase of first division in ascus. Sixteen chromosomes, many showing a longitudinal split, pass to each pole. × 3,000.

Fig. 16. Telophase of first division in ascus. × 3,000.

Fig. 17. Reorganization of daughter nuclei after first division. × 3,000.

Fig. 18. Prophase of second division in ascus, showing sixteen longitudinally split V-shaped chromosomes with widely divergent limbs. × 3,000.

Fig. 19. Second division in ascus. Individual chromosomes from several different nuclei. × 3,000. (*a*) Prophase. Longitudinally split V-shaped chromosomes with divergent limbs. (*b*) As (*a*), but limbs of chromosomes wrapped round each other. (*c*) Anaphase beginning. Separation at attachment region, distal limbs united. (*d*) Anaphase. Later stage than (*c*).

Fig. 20. Second division in ascus. Chromosomes on spindle. × 3,000.

Fig. 21. Second division. Late anaphase in almost polar view. Sixteen V-shaped chromosomes at each pole. × 3,000.

PLATE XXV

Fig. 22. Resting stage between second and third division in ascus. × 3,000.

Fig. 23. Early prophase of third division in ascus. Each chromosome shows two chromonemata. Only part of nucleus drawn. × 3,000.

Fig. 24. Late prophase of third division in ascus. Chromosomes show longitudinal split in limbs. Part of nucleus drawn. × 3,000.

Fig. 25. Metaphase of third division in ascus in polar view showing sixteen chromosomes. Anaphase separation of daughter chromosomes has started in two cases. × 3,000.

Fig. 26. The chromosomes which have started anaphase separation from nucleus in Fig. 25. The daughter chromosomes are beginning to move apart at the region of attachment to the spindle, but they are attached distally in one limb. × 3,000.

Fig. 27. Anaphase of third division in ascus showing sixteen daughter chromosomes directed towards each pole of the spindle. × 3,000.

Fig. 28. The chromosomes of the nucleus shown in Fig. 27 drawn separately showing shapes typical of the separation of two more or less V-shaped daughter chromosomes. × 3,000.

Fig. 29. Anaphase of third division showing similar stages as in two previous figures. *Only part of nucleus is drawn.* × 3,000.

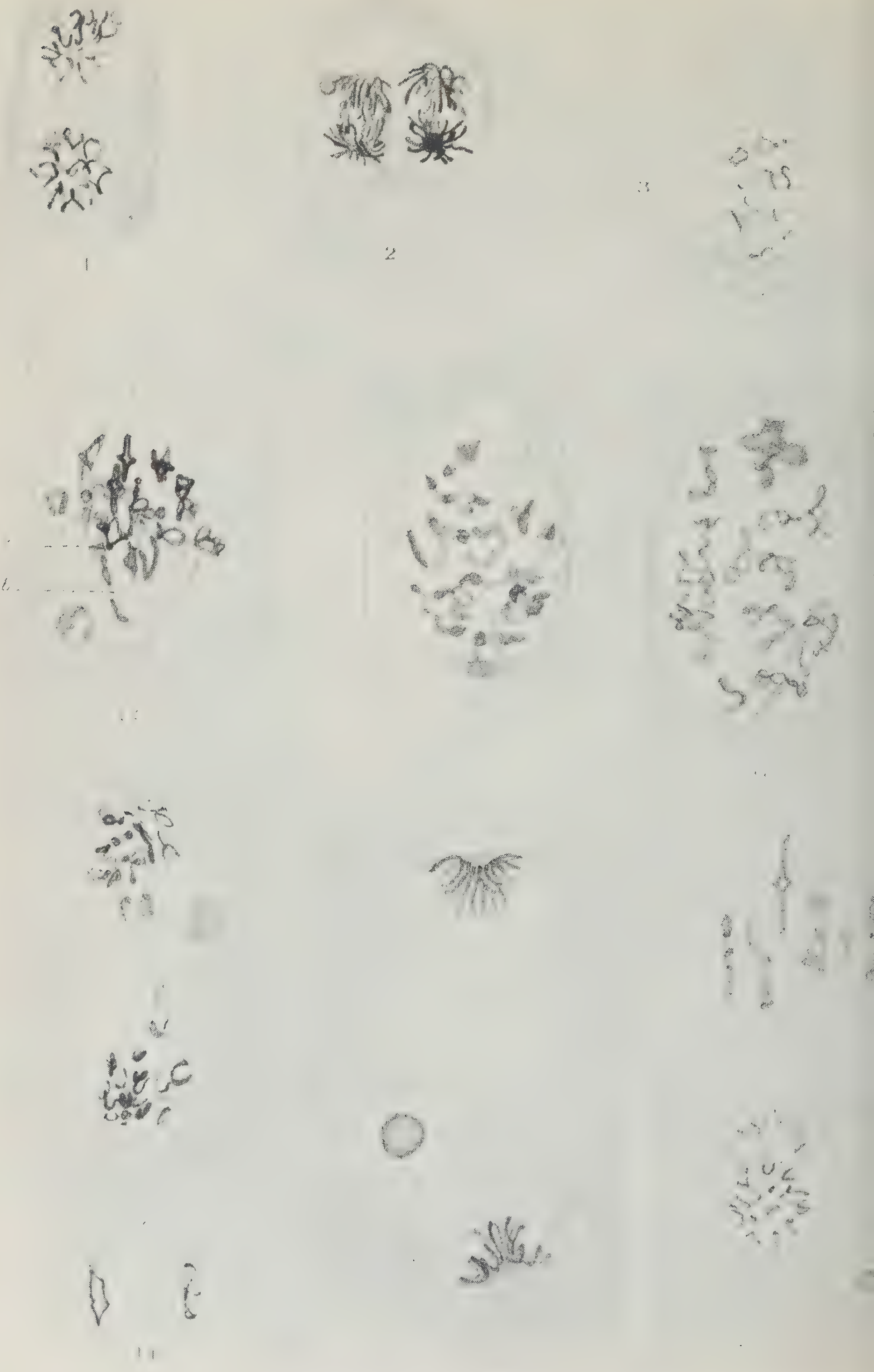
Fig. 30. Late anaphase of third division in ascus. The lower half of the nucleus shows about sixteen V-shaped chromosomes. × 3,000.

Fig. 31. Telophase of third division in ascus. Polar view. Only one daughter nucleus represented. Apices of sixteen V-shaped chromosomes can be counted. × 3,000.

Fig. 32. Abnormal ascus showing young akaryotic spore with refractive spot. × 1,000.

Fig. 33. Abnormal ascus showing an akaryotic spore and a tri-nucleate spore. × 1,000.

Fig. 34. Mitosis in vegetative nucleus from cell at base of ascocarp. × 3,000.



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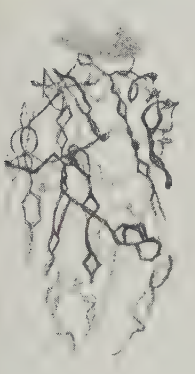

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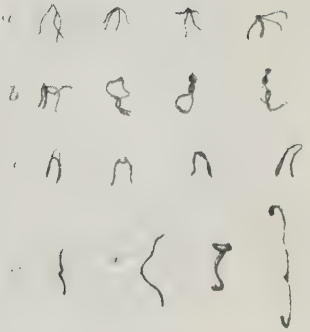

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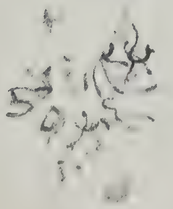

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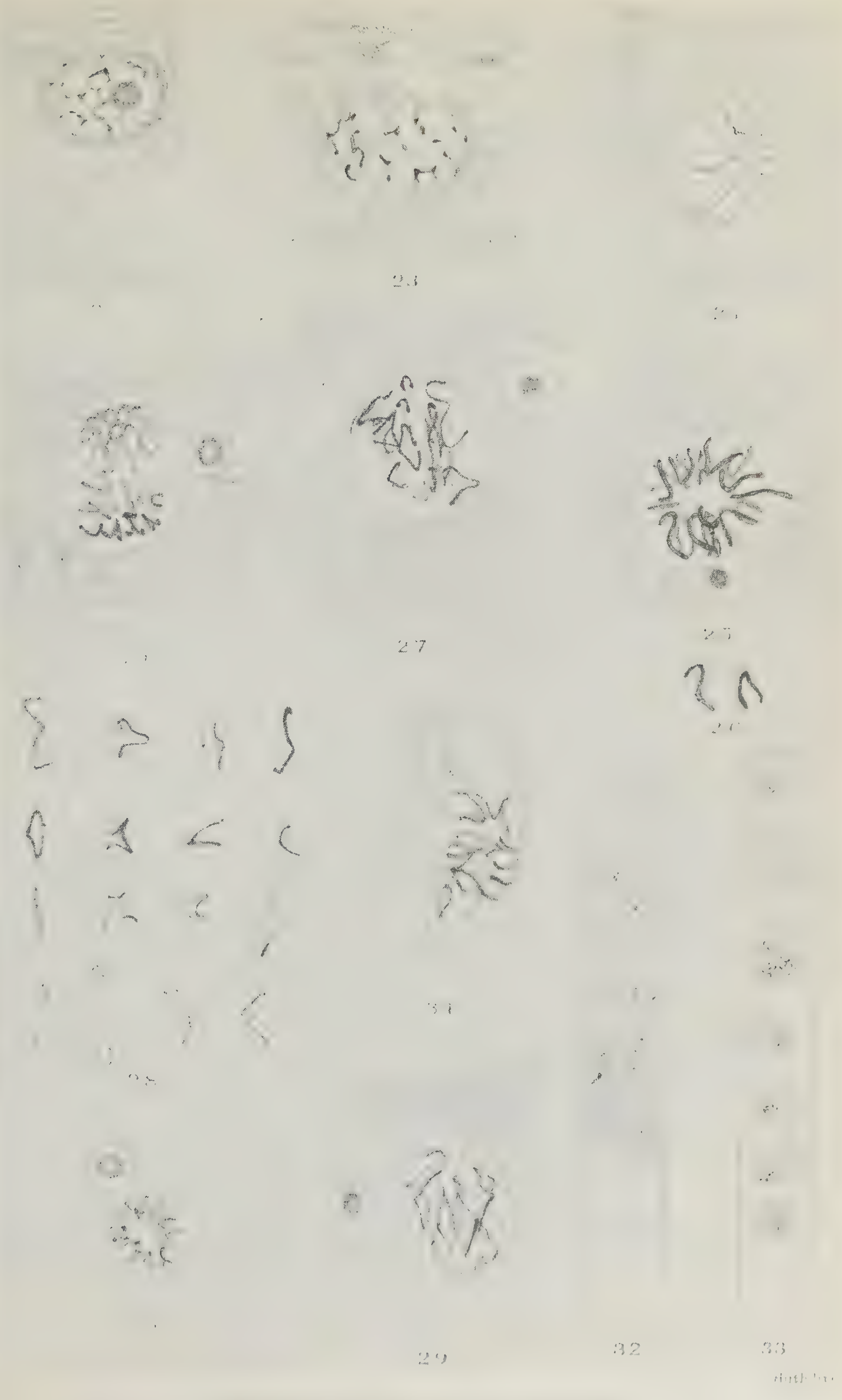

WILSON—NUCLEI OF PEZIZA.

The Accurate Definition of Leaf Shapes by Rectangular Coordinates

BY

R. MELVILLE

With two Figures in the Text

A SMALL number of terms descriptive of the forms of leaves and other plant organs is in common use among botanists. The meaning of such terms as ovate and lanceolate is generally understood, though there is often some difference in their application. Attempts have been made to standardize the terminology by the publication of definitions and illustrations, but it is almost impossible to describe in words the infinite variety of intermediate shapes. This paucity of descriptive terms leads to confusion and is a real difficulty in the critical study of variable genera and species. The difficulty is intensified when asymmetrical structures such as elm leaves are the subject of study.

It is obvious that any outline, however complicated, could be represented by a series of rectangular coordinates. The method would be cumbersome for very complex shapes, but the numerous forms approximating to ovate, for example, can be adequately defined by about twenty coordinates. For most purposes it is desirable to obtain means from the measurements of a number of leaves. The system of measuring therefore must be so designed that measurements made on leaves of different sizes shall be comparable. This object can be attained if the length of the lamina is always divided into the same number of equal parts, irrespective of size of the leaf, and breadths are measured in the same units. It is convenient to divide the lamina from base to tip into ten parts and measure co-ordinates of breadth as percentages of the length at intervals of ten per cent. of the length from the base up to the tip. The midrib is usually nearly straight and can be used as a line of reference from which to measure coordinates.

The two lateral flanges of the lamina are then dealt with separately, a procedure which enables the short and long sides of an asymmetrical leaf to be compared separately with the corresponding parts of other leaves. The mean coordinates for comparable leaves of one plant can be obtained and compared with similar data from another plant. The significance of any differences can be tested by statistical methods.

The following technique has been developed. The leaf, spray of foliage, or herbarium sheet is placed on a sheet of glass with a strong light below

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and is covered with a sheet of semi-transparent 'grease-proof' paper. The outline of the leaf and the position of the midrib are drawn with indian ink. In the case of serrate or dentate leaves the outline is drawn through the points of the teeth (Fig. 1). The outline drawing is placed in the carrier of an automatic focusing type of photographic enlarger and the projected image enlarged until its length extends across the ten squares of a rectangular scale of suitable size as indicated in the figure. The central line of the scale is placed along the midrib of the image and the breadths from the midrib to the periphery read at the intersection of each horizontal line with the leaf outline. The vertical lines make a scale of tenths of the length of the leaf.

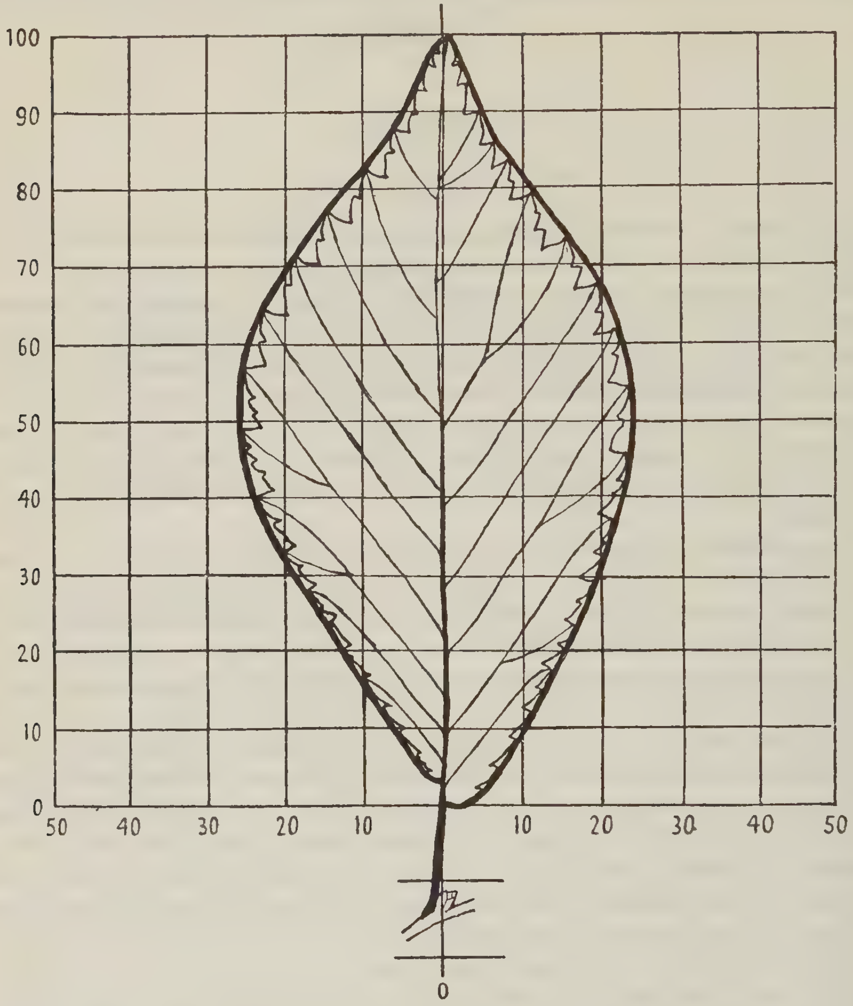

FIG. 1. Tracing and outline of a leaf of *Ulmus nitens* Moench superimposed on a rectangular scale

The scale is moved slightly sideways for the central line to coincide with the midrib for each reading when the midrib is curved. This reduces the error due to curvature of the midrib.

The technique outlined above is being applied to a study of the leaves of elms. The foliage in this genus is very variable and differs on a single tree according to position, whether on a normal shoot, leading shoot, proleptic shoot, epicormic shoot, or sucker. The short lateral shoots on normal branches bear the bulk of the leaves of a tree and also exhibit the smallest variation. The distal and subdistal leaves of such shoots can be used for a comparative study of the species and hybrids. As an example of the application of the method, the co-ordinates obtained from ten distal leaves taken at random from a single tree of the English Elm, *Ulmus procera* Salisb. are given in Table I. In this species the margin of the leaf meets the petiole at some distance above the lowest point of the lamina, and it is necessary to determine coordinates of length for the points of contact for each side as the leaf is asymmetric. These measurements are given in the second column of Table I. On the long side of the leaf a coordinate of breadth for the lowest point of the leaf is needed as well. By adopting the convention of writing the co-ordinate of length as the numerator of a fraction and that of breadth as the denominator the mean co-ordinates, taken from base to tip, for the distal leaves of this species can be written thus:

Short side 10/0, 10/4, 20/18, 30/27, 40/32, 50/34, 60/33, 70/29, 80/21, 90/8.
Long side 3/0, 0/6, 10/19, 20/26, 30/31, 40/33, 50/33, 60/31, 70/26, 80/18, 90/7.

The co-ordinates provide a concise and exact method of describing the shape of a leaf and, moreover, the shape can be reproduced quickly and easily by plotting the coordinates on squared paper. The length of the petiole can be given in the same units, for the above example 9 per cent. of the length of the lamina. The actual mean length, 7·7 cm., can then be used for converting any given figure into absolute terms.

The mean coordinates determined from ten leaves for distal and subdistal leaves of short shoots of the same tree of *U. procera*, together with the corresponding figures for another tree, believed to be a hybrid of this species, are given in Table II. It will be noticed that the distal leaves are narrower than subdistal leaves, a feature common to most elms. The long sides of corresponding leaves of *U. procera* and the presumptive hybrid do not differ appreciably in shape. The short sides of the leaves of the two trees are closely similar in the upper half, but the hybrid leaves are much narrower in the lower half, notably at 20 to 30 per cent. along their length from the base. These similarities and differences are clear from the graphs (Fig. 2) in which the ratio of the breadths of hybrid to *U. procera* leaves are plotted. The significance of the differences at 20 and 30 per cent. along the length on the short side of the leaves has been tested statistically by Student's method (Fisher, 1928). For distal leaves the numerical difference is 3 and for subdistal leaves 4 at both 20 and 30 per cent. along the length. The values of t

TABLE I

Coordinates, Petiole Ratio, and Length of Lamina of Distal Leaves of Ulmus procera *Salisb.*

Short Side. Co-ordinates of length.	*A*	0.	10.	20.	30.	40.	50.	60.	70.	80.	90.	Length of Lamina, cm.
Leaf No. 1	10/0	—	—	17	27	32	34	32	28	18	6	8·6
2	10/0	—	—	19	27	32	34	33	29	21	6	8·7
3	13/0	—	—	13	24	28	31	32	29	21	9	6·5
4	10/0	—	—	18	26	31	33	32	30	23	8	9·2
5	7/0	—	9	21	31	35	36	35	30	22	9	5·4
6	9/0	—	7	23	31	34	35	34	30	23	10	7·4
7	9/0	—	14	18	26	30	32	30	27	18	7	8·1
8	13/0	—	—	19	27	31	33	32	28	19	7	8·7
9	9/0	—	4	16	25	30	33	33	30	23	10	6·8
10	9/0	—	3	19	29	34	35	33	29	20	7	7·4
Means	9·9	—	3·7	18·3	27·3	31·7	33·6	32·6	29·0	20·8	7·9	7·68
Long Side.												Petiole length/ Lamina length
1	2/0	6	19	25	30	33	33	31	25	15	5	6
2	3/0	5	21	28	32	34	34	32	26	18	6	6
3	8/0	10	25	30	34	35	35	33	28	20	8	13
4	4/0	4	18	25	30	33	33	31	27	17	7	9
5	2/0	5	19	27	32	34	34	31	27	20	9	12
6	2/0	7	19	25	29	31	32	31	26	19	9	6
7	3/0	5	18	24	29	32	32	29	24	16	7	10
8	3/0	8	22	29	32	34	33	31	26	17	6	8
9	3/0	5	16	25	30	33	34	32	27	18	8	8
10	2/0	5	17	26	31	32	32	29	25	17	7	7
Means	3·2/0	6·0	19·4	26·4	30·9	33·1	33·2	31·0	26·1	17·7	7·2	8·5

Column *A*: Point of union of margin and petiole.

TABLE II

Mean Coordinates and Lengths of Distal and Subdistal Leaves of Ulmus procera *and a Related Elm*

												Length of Lamina, cm.
Distal Leaves.						Short Side.						
U. procera	10/0	10/4	20/18	30/27	40/32	50/34	60/33	70/29	80/21	90/8		7·7
Hybrid	8/0	10/4	20/15	30/24	40/30	50/33	60/32	70/29	80/21	90/8		6·2
						Long Side.						
U. procera	3/0	0/6	10/19	20/26	30/31	40/33	50/32	60/31	70/26	80/18	90/7	
Hybrid	2/0	0/4	10/19	20/25	30/30	40/32	50/33	60/31	70/26	80/18	90/8	
Subdistal Leaves.						Short Side.						
U. procera	11/0	—	20/21	30/30	40/34	50/36	60/35	70/31	80/22	90/10		6·2
Hybrid	9/0	10/3	20/17	30/26	40/31	50/34	60/33	70/29	80/21	90/10		5·6
						Long Side.						
U. procera	5/0	0/8	10/22	20/28	30/33	40/35	50/35	60/33	70/29	80/21	90/10	
Hybrid	4/0	0/7	10/23	20/29	30/33	40/35	50/35	60/33	70/29	80/22	90/10	

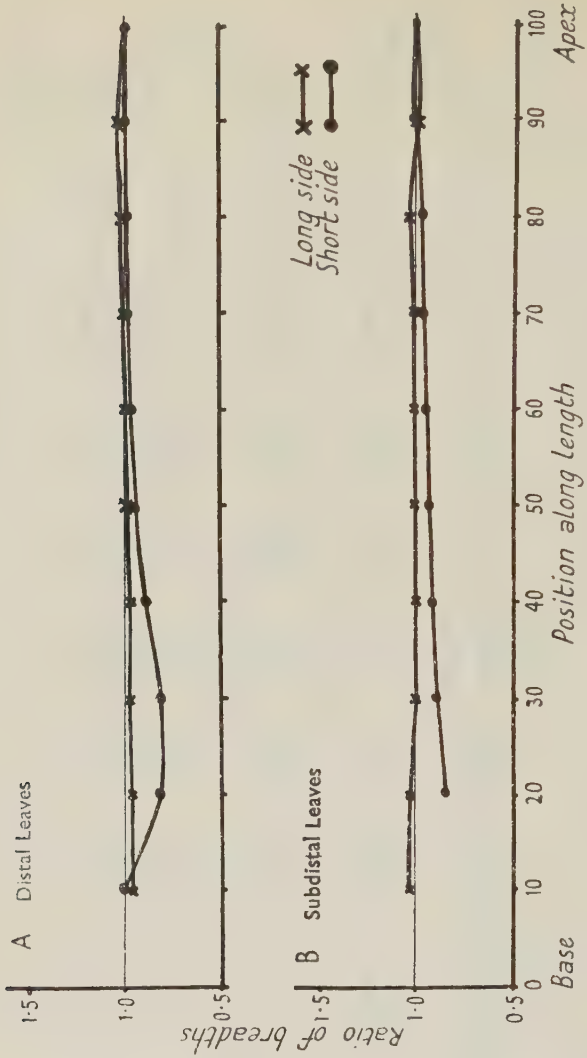

FIG. 2. Ratio of breadth of *Ulmus procera* to hybrid leaves

calculated from the data are for distals at 20 per cent., 2·675; at 30 per cent., 2·715; for subdistals at 20 per cent., 0·694; at 30 per cent., 3·685: for a probability of 0·05 *t* is 2·101.

The differences between the pairs of means tested, therefore, are significant in three cases, but when tested by the same method the eight individual means are all highly significant. It is evident from this result that for most purposes ten leaves are sufficient for calculating the mean coordinates.

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ADDENDUM

The method of describing leaf shapes by rectangular coordinates could be adopted with advantage in monographs and other floristic works where there is a need for accurate definition of closely related shapes. Where simple shapes are concerned, the method could be applied without modification, but difficulties are likely to occur when dealing with leaves having an involved outline or when the surface does not approximate to a plane. Such cases would call for special treatment and may prove to be beyond the scope of the method. However, there is no doubt that a large variety of shapes could be described in comparable terms in this way, provided the most proximal point of an organ is always placed on the base line of the scale, the most distal point on the 100 per cent. line, and its axis along the central line. For leaves, it is convenient to place the proximal point of the lamina on the base line without regard to the place of insertion of the petiole, which then may be 50 per cent. or more along the length in some sagittate leaves. In this way, any need for the use of plus and minus signs can be avoided. The series of coordinates for each half will begin in all cases at the point of insertion of the lamina on the petiole and pass in sequence round the periphery to the tip of the axis.

Studies of the Physiology of Coffea arabica

II. Stomatal Movements in Relation to Photosynthesis under Natural Conditions

BY

F. J. NUTMAN

(*East African Agricultural Research Station, Amani, Tanganyika Territory*)

With eight Figures in the Text

IN a previous communication (Nutman, 1937) examples have been given of the daily march of assimilation of *Coffea arabica* leaves in the field, showing that there is a marked midday drop in assimilation rates when the leaves are exposed to direct sunlight, but not when they are shaded, nor during cloudy weather. It was concluded that this drop in assimilation was not due to the water relations of the leaf or of the plant as a whole, nor to an excessive accumulation of assimilates in the leaf, nor to a solarization effect. It was suggested that stomatal changes might explain the observed phenomena, and, if so, stomatal aperture should be directly correlated with light at low intensities inversely correlated at high intensities.

The object of this paper is to record the type of stomatal movement in leaves of *C. arabica* in the field, and to show that these movements will suffice to explain the observed variations in assimilation rates.

Apparatus and Methods

The work here reported was begun in 1929 and has been continued at irregular intervals until 1936. Early records were obtained with a Knight porometer (Knight, 1922) in conjunction with a recording drum and an electrically operated pen. These were at first checked by the Lloyd alcohol method (Lloyd, 1908), but since no serious discrepancy was ever noted this was discontinued. The resistance porometer of Gregory and Pearse (1934) enabled more useful records to be obtained. All the later records were made by this instrument, both in its original form and with the self-recording modification that I have previously described (Nutman, 1937).

Total solar radiation was measured by a Callendar sunshine recorder, and by a Gorczinsky solarimeter, the latter being used in the field. Both these instruments integrate radiation in terms of heat, so that, from the point of view of this investigation they give disproportionate weight to the infra-red rays which have been shown (Sayre, 1929) to be ineffective in causing stomatal opening.

[Annals of Botany, N.S. Vol. I, No. 4, October 1937.]

It may be noted that when plants are in full sunshine the radiation falling on them is the same as that recorded on a nearby instrument; whereas when the plant and recorder are both under a natural form of shade, with its concomitant irregular sun-flecks, the readings only approximate to the radiation falling on the leaf. Under these conditions close concordance is not to be expected.

THE RECORDS OBTAINED

During the past seven years several hundred records of stomatal movements throughout the day have been obtained at all seasons of the year and in three different localities, at Amani in the East Usambaras, at Usa River on the southern slopes of Meru, and at Burka on the western slopes of the same mountain. Except where otherwise stated, I have worked on mature leaves of trees grown under such conditions that at no time was wilting of the youngest leaves observed—where water-strain in the coffee plant first makes itself obvious. Care was taken that both the leaves measured and the recording surface of the radiation instrument were always horizontal. From the records thus obtained the eight figures reproduced have been selected as typical of the stomatal behaviour under the conditions described.

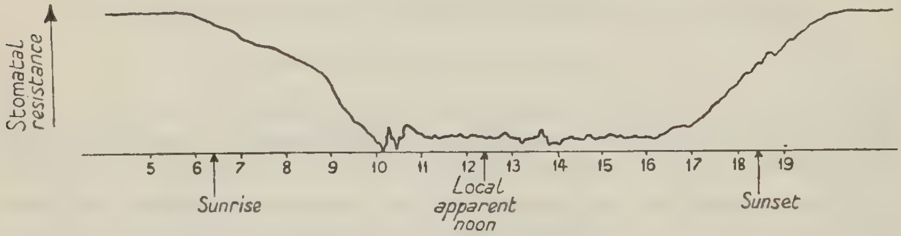

FIG. 1. Stomatal resistance of a horizontal coffee leaf throughout an overcast day. Amani, 1935.

Fig. 1 is a record by the recording resistance porometer at Amani during a day of persistent cloud. The stomata are open for the greater part of the day.

Fig. 2 is also from Amani by the same instrument. It extends over three days, which were all dull in the mornings. The first was clear from 11 h. to 13 h.,[1] but considerable cloud occurred during the remainder of the day. The second was similar, but with rather less cloud during the afternoon. The third was duller than the others during the morning, with variable weather during the midday hours, and with but little cloud during the afternoon.

Fig. 3 is a similar chart, also from Amani, during a fairly clear day, the midday hours being almost cloudless. For two periods, shown by hatching on the chart, the plant was shaded by one thickness of unbleached calico.

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[1] Throughout this paper times are referred to as Tanganyika standard time, this being about half an hour ahead of approximate sun time.

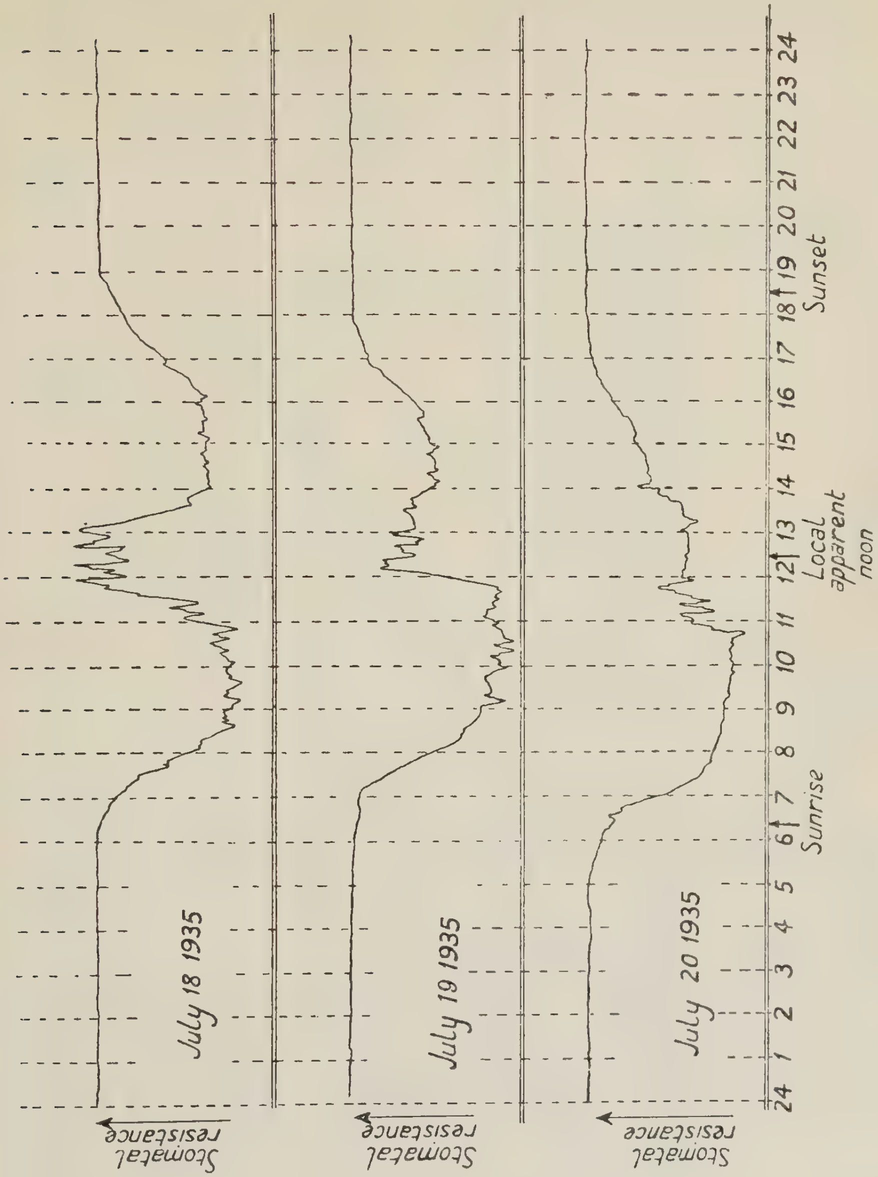

FIG. 2. Stomatal resistances of one leaf of *C. arabica* over three days. For description see text.

Fig. 4, also from Amani, is by the Knight porometer. The unbroken line represents the stomatal aperture from 10 h. 45 m. to 12 h. 45 m., and the broken line the total solar radiation as given by a Callendar sunshine recorder over the same period. It will be seen that the relation between the two is more or less a direct one for low values of radiation (below 0·9 gm. cals./cm.^{2}/min.) but it tends to be reversed at higher values.

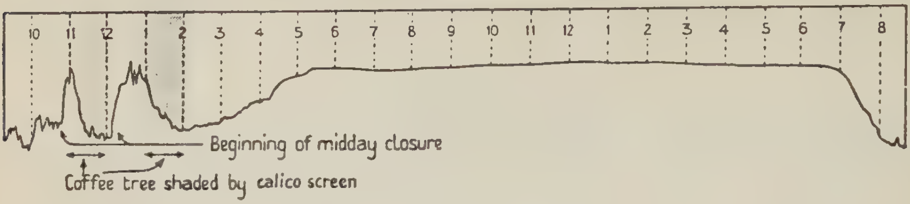

FIG. 3. For description see text.

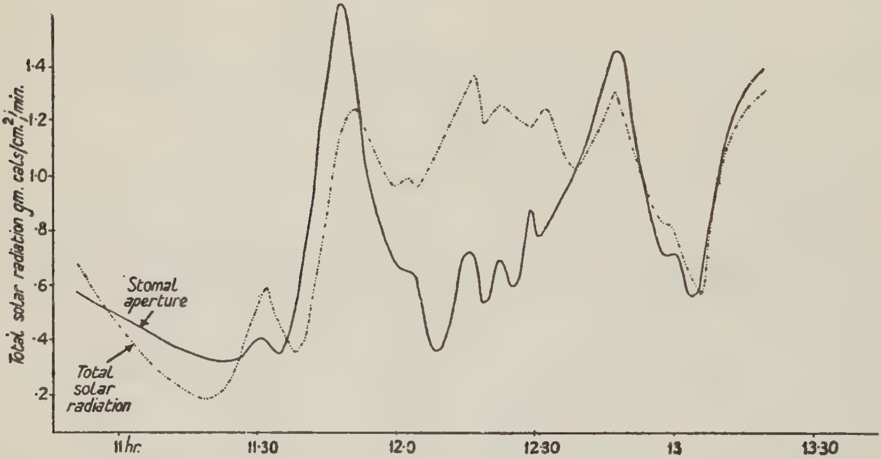

FIG. 4. Stomatal aperture by Knight porometer and total solar radiation by Callendar sunshine recorder from 10 h. 45 m. to 13 h. 15 m. on Oct. 14, 1930, at Amani.

Fig. 5 is a chart obtained near Arusha on the south-west slopes of Mount Meru. Simultaneous readings of the stomatal resistance of unshaded leaves as given by the resistance porometer and of solar radiation as given by a Gorczinsky solarimeter were taken at intervals of one minute throughout the day. Stomatal resistance started to fall soon after 7 h. 30 m. and by 9 h. the stomata were widely open. From 8 h. 45 m. to 11 h. 15 m. minor fluctuations in the radiation were mirrored to a remarkable degree by the stomatal behaviour, and during this period the relationship between stomatal aperture and radiation was obviously a direct one. At 11 h. 15 m. a burst of sunshine lasting for nearly fifteen minutes was recorded, and after a transitory opening stomatal resistance rose. From 11 h. 45 m. during a period of dull weather it fell. With increasing light intensity the stomata soon closed, and remained

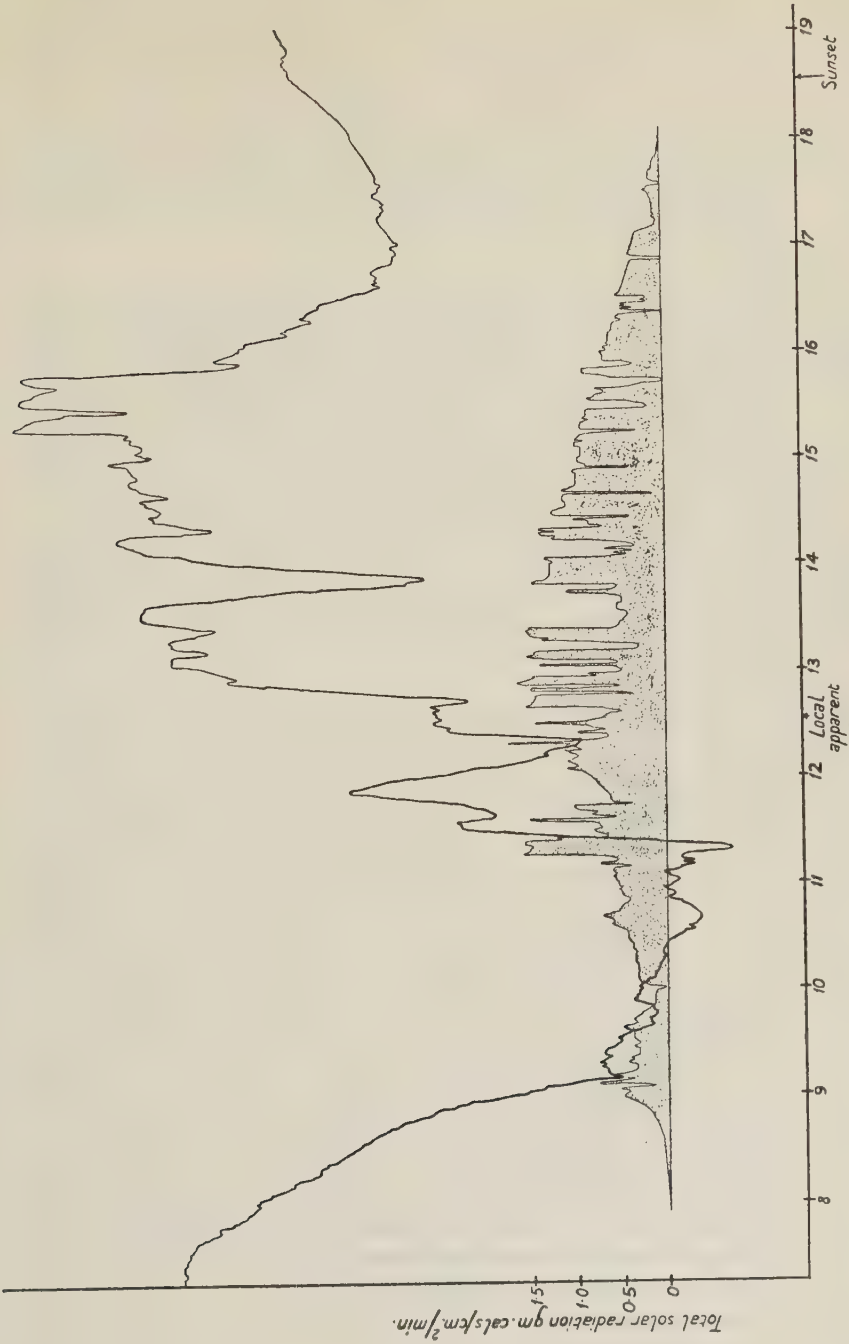

FIG. 5. Stomatal resistance of unshaded coffee leaf (above) and total solar radiation by Gorczinsky solarimeter (below) from 6 h. 15 m. to 19 h. on Aug. 26, 1935, at Burka.

closed until 16 h. save for a tendency to open at 13 h. 35 m. in response to a cloudy period lasting from 13 h. 25 m. to 13 h. 50 m. Between 11 h. 15 m. and 16 h. every peak on the radiation curve is reflected, almost immediately, by a tendency for the stomata to close, every depression below about 0·7 gm. cals./cm.^{2}/min., even those lasting but a minute or so, by a tendency to open. After 16 h., though still in full sunlight, the stomata reopened, owing, doubtless, to the reduced amount of radiation falling on the leaf as the sun declined. They closed with the onset of darkness at about 19 h., although their resistance did not become infinitely large until after 22 h. (not shown on the figure).

Fig. 6, obtained with the same apparatus, is from a tree growing under Erythrina shade at Usa River. A break in the canopy allowed full sunlight to fall on both the leaf and the Gorczinsky solarimeter from about 14 h. 20 m. till 16 h. 20 m. During the rest of the day the distribution of sun-flecks between the leaf and the solarimeter was of course unequal and variable. Throughout the day variable cloud occurred, which did not appreciably reduce the solar radiation recorded under the natural shade by the particular instrument used. The values obtained were low throughout the day, save when sunlight penetrated through the gap in the canopy. The stomata opened during the early morning, and remained open till 15 h. 55 m., with minor fluctuations, correlated no doubt with sun-flecks and cloud. So soon as full sunlight fell on the leaf, however, the stomata closed, and did not reopen till 16 h. 25 m.

Fig. 7 is a similar record for another leaf of the same tree two days later. After the porometer had been attached to the leaf all the other leaves on the tree were removed. It will be seen that the general lines of the graph are similar to those of the previous day, with a prolonged period of opening, and closure during the time when the sun was shining on the leaf through the gap in the canopy. But, since occasional cloud passed during the period 14 h. 20 m. to 15 h. 45 m. the contrast between the 'sun' and the 'shade' portions of the curve is not so well marked as in Fig. 6. During the 'sun' period, marked falls in stomatal resistance are shown corresponding to periods of cloudiness on the solar radiation curve at the same times.

Fig. 8, also obtained at Usa River by the same apparatus, shows the stomatal movements of a leaf attached to a tree suffering from water-strain which was apparent in the youngest leaves, although the older ones, including the one to which the porometer was attached, remained apparently turgid. The tree, although at the time healthy and with normal green leaves, had probably suffered from slight dieback in the past. This has been shown (Nutman, 1933) to restrict the absorbing area of the roots. The dry conditions of the time of year, together with the probable root limitation, would suffice to explain the slight wilting of the particular tree.

Stomatal closure began before 11 h. and was complete by 13 h. No subsequent opening took place that day.

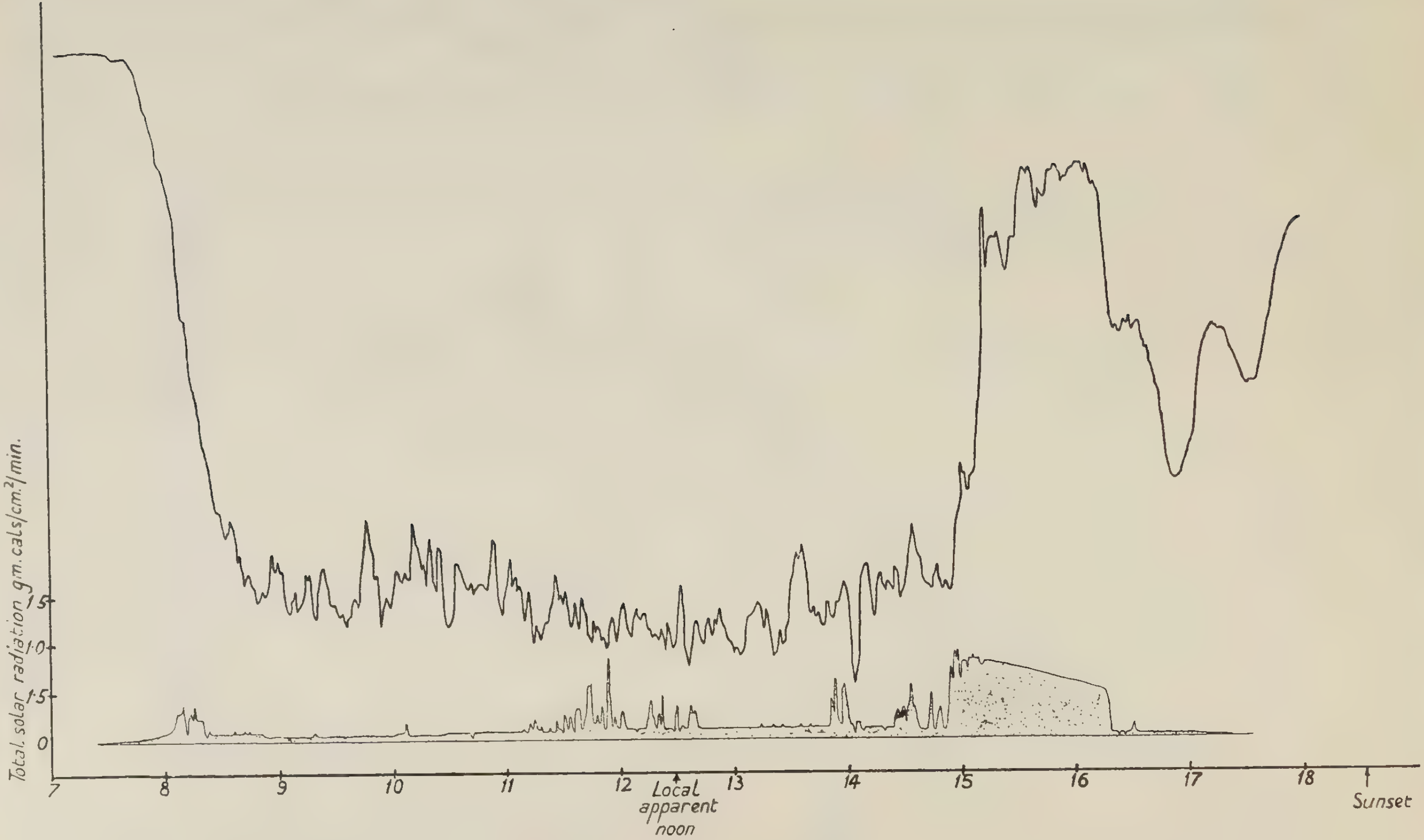

FIG. 6. Stomatal resistance of coffee leaf (above) and total solar radiation by Gorczinsky solarimeter (below) under natural shade; from 7 h. to 18 h. on Sept. 4, 1935, at Usa River.

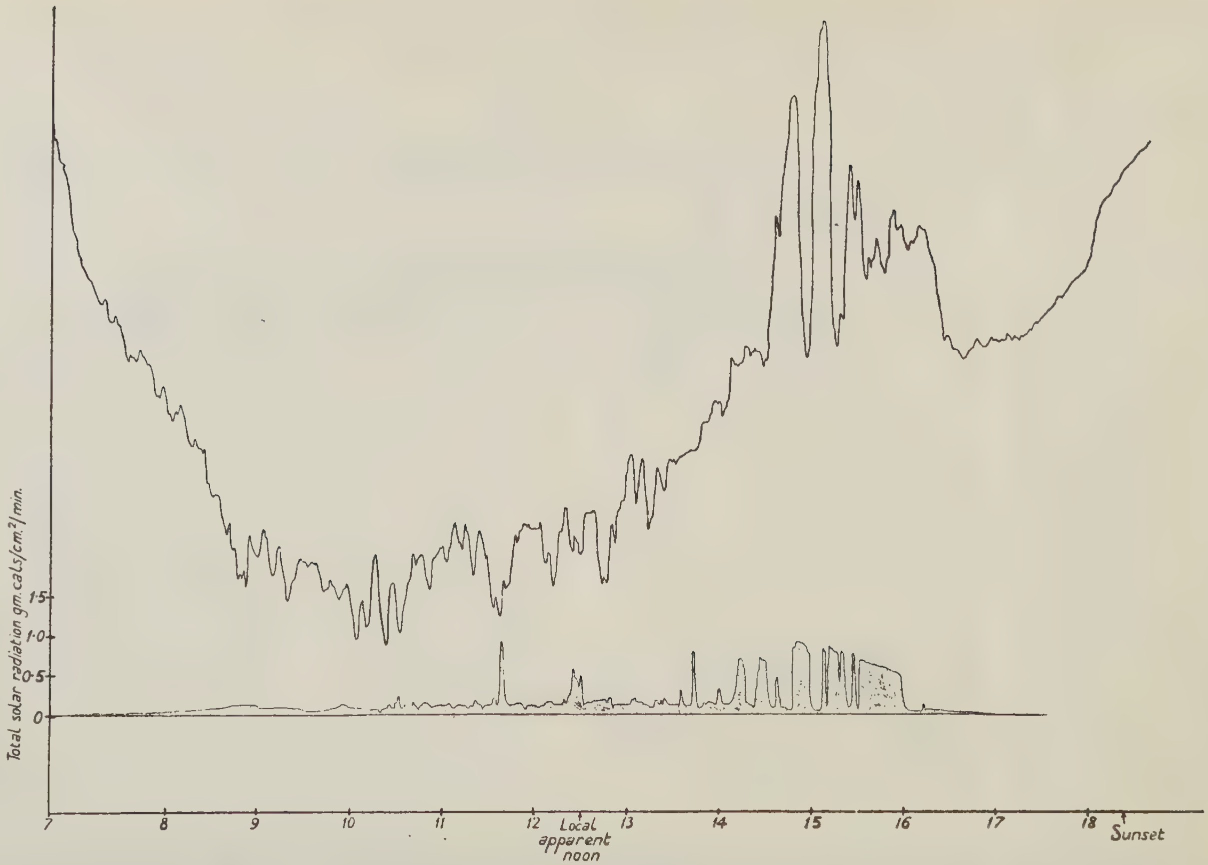

FIG. 7. Stomatal resistance of coffee leaf (above) and total solar radiation by Gorczinsky solarimeter (below) under natural shade; from 6 h. 45 m. to 18 h. 30 m. on Sept. 6, 1935, at Usa River. Except for this leaf the coffee tree had been defoliated.

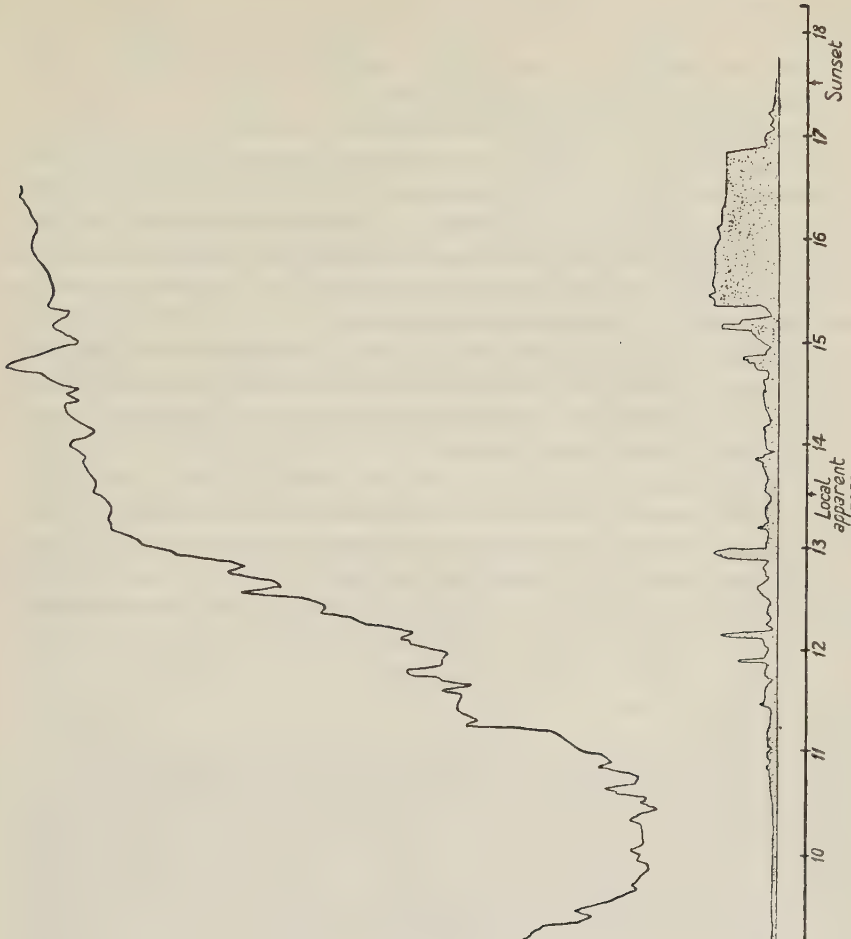

FIG. 8. Stomatal resistance of a coffee leaf (above) and total solar radiation by Gorczinsky solarimeter (below) under natural shade; from 7 h. 0 m. to 17 h. 30 m. on Oct. 6, 1935 at Usa River. The coffee tree showed signs of water-strain.

RESULTS

From the foregoing data the following results emerge:

1. The stomata of *C. arabica* remain open throughout the day during dull weather or under shade.

2. When direct sunlight falls on the leaf between the hours of 10 (exceptionally 9 h. 20 m.) and 16 the stomata close.

3. Reopening can be induced at any moment by shading.

4. From a consideration of all the available data obtained with the Gorczinsky solarimeter, of which the records reproduced form only a small part, stomatal aperture appears to be directly correlated with radiation intensity up to about 0·7 gm. cals./cm.^{2}/ min. and inversely correlated at intensities above about 0·9 gm. cals./cm.^{2}/min. Records by the Callendar sunshine recorder are neither so numerous nor so accurate, but from the results obtained therefrom the radiation intensity at which the change from direct to inverse correlation takes place is in the region of 0·9 gm. cals./cm.^{2}/min.

5. The time-lag between the light-stimulus and the beginning of movement does not normally exceed three minutes.

6. The diurnal march of stomatal movements in a single leaf is not appreciably affected when all the other leaves are removed from the tree.

7. Under unfavourable conditions, where signs of wilting are to be seen in the youngest leaves, the stomata of other leaves on the same tree close before midday, and remain closed for the rest of the day, even under shade.

DISCUSSION

The midday closure.

The midday closure of stomata under high light intensity and conditions favouring transpiration has been frequently observed. Loftfield (1921) came to the conclusion that the midday closure was a response to incipient wilting; that the effect of light, which normally operates to cause stomatal opening, was nullified by other factors reducing the water content of the leaf. He says: 'Part of the water present in the leaf at the start (of a day) is a working margin, and the stomata do not close until it is gone. If the roots can keep up a sufficient rate of supply the working margin is not wholly lost and the stomatal movement is of the normal light-induced type. If the margin is lost, however, the stomata close until it is regained, at least in part.'

This view has been generally accepted by botanists. For example, Maximov (1929) commenting on Linsbauer's finding that an optimum light intensity might exist for stomatal opening, says: 'This may be so in shade plants which close their stomata under the influence of strong light. It is possible, however, that in this case the effect is partly due to the water content of the leaf, a factor which also influences stomatal movement.'

In considering the data put forward in the present paper it must always be borne in mind that none of the species with which Loftfield worked can be regarded as a typical shade plant. The natural habitat of coffee is the understory of evergreen forest in a low latitude but a high elevation, and its assimilation proceeds best in shade. The evidence is against the extension of Loftfield's explanation of stomatal movements to coffee, for the following reasons:

1. When a plant is defoliated except for a single leaf the water supply available for that leaf is relatively unlimited. Yet stomatal closure in response to increased radiation is as complete and rapid as in a leaf on an undefoliated tree.

2. On the hypothesis that the stomata close when the water content of the leaf falls to a critical level and are not able to reopen until its margin of safety has been regained, reopening of the stomata can only follow closure after a minimum time. This time should be approximately constant under similar conditions. But in my observations opening has followed closure at most variable intervals, from a few seconds to several hours, and moreover the intervals have consistently agreed with light fluctuations.

3. The speed at which movements take place, with a time-lag of normally less than three minutes, renders it unlikely that water-strain conditions them. For example, in Fig. 3 at 12 h. the stomata were fully open and *ex hypothesi* the leaf contained a plethora of water; but on exposure to the sun closure began at once, and was complete in about fifteen minutes.

4. When the whole coffee bush is affected by the midday closure, due, on Loftfield's hypothesis, to lack of water in all the leaves, we can induce a single leaf to reopen its stomata without delay by shading it. *Ex hypothesi* this could only mean that it had been enabled instantaneously to regain its margin of safety in competition with the five thousand or so other leaves on the plant.

5. The results in this paper with one exception (Fig. 8) are all from plants growing under such conditions that no reduction in turgor was to be seen at any time in the youngest leaves. Under conditions where these leaves do show wilting, however, I have observed midday closure and slow recovery, uncorrelated with light fluctuations, in non-wilting leaves of the same tree. Thus, in Fig. 8, no recovery took place for the remainder of the day. One can, without hesitation, attribute this type of movement to changes in turgor.

One is therefore forced to the conclusion that radiation exercises a twofold action on the stomata of coffee: that at low intensities it causes opening, while at high and presumably supra-optimal intensities it causes closure; and that the closure at high intensities is not normally due to changes in the water content of the leaf, although under appropriate conditions closure can be induced by water shortage.

The relation between stomatal aperture and photosynthesis.

It has been generally accepted that stomatal movements can control assimilation by varying the rate at which carbon dioxide is supplied to the chloroplasts. This assumption has received support from the work of Maskell (1928) who showed that in *Prunus lauro-cerasus* the photosynthetic rate is not solely conditioned by temperature, light intensity, and carbon dioxide supply, but that stomatal control is appreciable. Mitchell (1936), however, has recently produced evidence that in tomato and *Pelargonium* the assimilation rate does not appreciably fall when the plants are placed in conditions demonstrated to cause stomatal closure.

Carbon dioxide can enter the leaf by diffusion through the stomata and through the cuticle. It is apparent that the amount of control exercised by the stomata will depend on the diffusion rate through the cuticle. If sufficient carbon dioxide for maximum assimilation under given conditions can enter in this manner, assimilation must be independent of stomatal aperture, whereas, if cuticular diffusion is small control by the stomata must be considerable. Coffee and cherry-laurel both have coriacious leaves. It is improbable that appreciable diffusion can take place through the well-developed cuticle of these plants, whereas those worked on by Mitchell have membranous leaves. Cuticular diffusion in these, especially with air 'slightly enriched with carbon dioxide' might well be appreciable.

I have previously suggested that the observed variations in assimilation rates of coffee in the field might be explained by changes in stomatal aperture, and give below, in the form of quotations, a brief summary of observed assimilation phenomena:

1. 'During cloudy weather the assimilation remains at a fairly constant value during most of the daylight hours.'
2. 'In the sun . . . the rates show a large depression during the midday hours.'
3. 'The shading of a leaf assimilating at zero rate suffices to bring about an immediate increase in assimilation.'
4. 'Assimilation rates appear to vary directly with light intensities when these are low, but inversely at high light intensities.'
5. 'Under all conditions of light the time-lag between a change in light and change in assimilation rate is less than two minutes.'
6. 'When the whole of the leaves of a tree, save only the experimental leaf, are covered by an opaque cloth . . . the behaviour of the leaf is in no way different from that when the other leaves . . . are exposed to external conditions.'

It will be observed that the assimilation phenomena, as summarized above, run parallel, step by step and with the closest exactitude, with the stomatal behaviour as described under paras. 1–6 of 'results'.

It is realized that the above does not form a completely logical proof of the controlling action of the stomata, since the data do not rule out the possibility that the two sets of phenomena (stomatal aperture and assimilation rate) may be each independently affected by some factor, or by some secondary cause itself affected by the factor. I find myself unable to suggest what such a factor might be, and consider that there is little reasonable doubt but that stomatal control of assimilation is effective in coffee.

SUMMARY

1. Typical examples of the march of stomatal movements of *Coffea arabica* are presented for a number of conditions.

2. Evidence is given leading to the conclusion that the marked midday closure of stomata on sunny days is due to the direct action of radiation on the stomata, and not to the water relations of the leaf. Thus, for coffee, light in excess of a value of about 0·9 gm. cals./cm.^{2}/min. leads to stomatal closure.

3. So numerous are the points of similarity between the movements of the stomata and changes in photosynthetic rate that the variations in assimilation can adequately be explained by the hypothesis of stomatal control, although the nature of the data precludes logical proof of this hypothesis.

ACKNOWLEDGEMENTS

I have pleasure in recording my thanks to Mr. F. A. F. Schmid of Burka, and Mr. C. Redfearn, of Usa River, for the facilities afforded me on their estates. My thanks are also due to Mr. R. E. Moreau, of this Station, for valuable and constructive textual criticism.

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Morphology and Orientation of the Embryo of Isoetes

BY

CHARLES LA MOTTE

Department of Biology, Agricultural and Mechanical College of Texas, College Station, Texas)

With Plate XXVI and twenty Figures in the Text

INTRODUCTION

THE literature on the embryogeny of Isoetes presents an outstanding example of the need for reinvestigation of some of the work of eminent investigators and for reconsideration of their interpretations. Campbell (1891) published the results of an investigation of the embryo of *I. echinospora* Dur., var. *Braunii* (Dur.) Engelmos, together with his interpretation of the observed developmental stages, and to the present time his interpretations have remained almost completely unchallenged, in spite of lack of agreement in certain details with the earlier observations of Hofmeister (1855) and Kienitz-Gerloff (1881). Several writers (Wilson-Smith, 1900; Liebig, 1931; and others) have confirmed certain of Campbell's observations, but such confirmation seems in every instance to apply only to advanced stages of the embryo rather than to the earlier critical stages.

There are perhaps two chief reasons for lack of thorough reinvestigation of the morphology of the early stages of the Isoetes embryo. In the first place, no attempt has been made to orient the embryos for sectioning in predetermined planes. The necessity of such orientation is obvious when one considers the bilateral symmetry of the embryo and the lack of a conspicuous suspensor to aid in positive identification of hypobasal and epibasal portions. In the second place, a study of advanced embryos suggests an exoscopic embryogeny and thus seems to verify the generally accepted interpretations based on Campbell's descriptions. But, as expressed by Liebig (1931, p. 350):

> 'Zur Entwicklung des Embryo gibt es verschiedene Figuren in der Literatur, doch fehlen in allen die genetischen Linien. Es sind auch nicht so viele Stadien in so lückenloser Folge vorhanden, als dass man die Linien finden und einzeichnen könnte.'

Recently the writer (La Motte, 1933) gave an account of the shifting orientation of the embryo of *I. lithophila* Pfeiffer and advanced the suggestion that the apex of the embryo may actually be derived from the lower quadrants rather than from the quadrants nearest the neck of the archegonium; in other words, that the embryo is probably endoscopic and hence similar in this

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respect to the embryos of the Lycopodiaceae and the Selaginellaceae. But the exact origin of parts of the embryo could not, with the material then at hand, be determined in sufficient detail to be conclusive. Furthermore, as was pointed out by Professor Bower in personal correspondence with the writer and again in his 'Primitive Land Plants' (Bower, 1935, p. 283), no consideration was given to the part played by gravity in determining the observed shift in orientation described in the earlier paper.

The purpose of the investigation herein reported has been, therefore, to make a detailed study of the early embryo under controlled conditions which would reveal the possible effect of gravity upon its form and orientation. And, fortunately, the technique developed for orientation of the megagametophytes in fixed positions made an accurate determination of embryological details possible.

MATERIALS AND METHODS

Four species of Isoetes were used in this investigation: *I. melanopoda*, *I. Engelmanni*, *I. japonica*, and *I. lithophila*. The last species was most intensively studied.

Plants and spores of *I. lithophila* and of *I. melanopoda* were collected in Texas by the writer; *I. Engelmanni* material was sent from Ithaca, New York, by Professor Eames; and spores of *I. japonica* were sent from Japan from the University of Tokyo.

The spores of *I. lithophila*, *I. melanopoda*, and *I. Engelmanni* were germinated by placing them in Syracuse watch-glasses and covering them with water which had stood for several days on soil from the native habitats of the plants. In order to reduce evaporation, these culture dishes were kept under a bell jar over water during the germination period. The spores of *I. japonica* were sent to the writer in vials of water, and the gametophytes began to reach maturity very soon after they were received.

As the megagametophytes reached maturity they were picked out individually with tweezers and placed in fixed positions for fertilization and subsequent development of the embryos. To accomplish this, strips of heavy bond typewriting paper were attached to glass slides, usually with rubber bands, and then melted plain agar (3 per cent.) was applied with a small brush to form a layer 1–2 mm. thick near one edge of the slide (Text-fig. 1). It was found best to scrape the paper lightly with a scalpel to loosen fibres and make sure that the agar would become firmly attached. Also, the water used as a solvent for the agar was the same as that used for germinating the spores. After the agar solidified, the slides were placed on edge in a staining dish containing enough water (again, the 'nutrient solution') to prevent drying of the agar.

It was found best to select megagametophytes whose spore coats had just cracked open and which were thus most likely to be ready for fertilization of the first-formed archegonia. Since the first archegonia are almost always centrally located in the tri-radiate apical exposure of the female gametophytes, chances of obtaining median sections of the subsequent embryos are greatly increased. The gametophytes were lined up in groups of five to ten in furrows gouged out of the agar with a flat-pointed needle just slightly narrower than the average diameter of the spores. The elasticity of the agar is such that the megagametophytes are held firmly in place once they are pushed into the trench to such depth that the equatorial ridges of the spore coats are just below the surface. The apical surfaces of the plants were thus left entirely exposed. Each slide was then orientated in such a way as to place the prothallia in the particular position desired and water containing numerous microgametophytes was added. The containers were then wrapped in heavy black cloth and placed in a dark closet to be left for the period desired; or in certain instances the slides were attached to a clinostat and rotated from the time the male gametophytes were added until the embryo leaf-tips emerged.

When the material was intended for sectioning, the agar strips were cut into portions 1 cm. or less in length, with one edge cut close to the row of gametophytes to serve as a marker in recording the orientation; and then, after the desired interval of time had elapsed, these were removed from the paper-covered slide with a thin knife and placed in the killing fluid. The agar hardens in the killing fluid and the gametophytes are then held in place more securely than ever for subsequent dehydration and infiltration.

Several killing fluids were used, but a modified Licent's fluid (chromic acid, 1 gm.; glacial acetic, 10 c.c.; commercial formalin, 30 c.c.; water, 160 c.c.) seemed to give the best results and the others were abandoned.

Several methods of dehydration and infiltration were employed, including the use of ethyl alcohol, tertiary-butyl alcohol, xylol, and chloroform. The best results were obtained by placing the material in diffusion shells for dehydration and leaving it for twenty-four hours in each of the 70, 95, and 100 per cent. ethyl alcohols. The material was then removed from the diffusion shells and run up through an alcohol-xylol series of 2½, 5, 7½, 12½, 25, 37½, 50, 62½, 75, 87½, 95, 100 per cent. xylol, three to twelve hours in each. It was then carried through very gradually from the infiltration agent into pure paraffin. Most of the imbedded material was sectioned to thicknesses of 5 to 10 μ. Of the several stains employed Delafield's haematoxylin proved to be the most suitable for histological details of early embryonic stages.

For *in toto* mounts the embryos were dissected out in the living condition. To accomplish the dissection the selected gametophytes were put into a watch-glass partly filled with some of the 'nutrient solution' in which the plants had been growing and which had been filtered to remove all debris. Then the three apical portions of the outer spore coat of each gametophyte were removed and, while the basal portion (still enclosed within that portion of the spore coat) was held with fine-pointed tweezers, a sharp, flat-pointed needle was used to lift off the firmer apical tissue. Frequently, then, the embryo would float out and settle to the bottom of the watch-glass, but if it should remain partly imbedded in the larger basal portion a slight squeeze with the tweezers would usually force it out. Very young embryos usually remained in the apical portions and were dislodged by scraping the tissues lightly with the flat point of the needle.

After the embryos were isolated from the gametophytic tissues they were immediately killed in either formalin-acetic alcohol or Licent's fluid. Then, after remaining in the killing fluid for twenty-four to forty-eight hours, they were transferred through several changes of water during a period of twenty-four hours, stained in Delafield's haematoxylin for three minutes, dehydrated in a series of alcohols, carried through a graded series into xylol or through several steps into pure clove oil, and finally mounted in balsam. All transfers were made with a pipette similar to that recommended by Lotze (1935) for handling individual protozoa.

Approximately 5,000 megagametophytes were arranged on agar for observation in the living condition and for sectioning. Of this total the recorded observations involved approximately the following numbers of prothallia and embryos:

300 in stationary horizontal position, producing 120 embryos.
200 in upright vertical position, producing 150 embryos.
200 in inverted vertical position, producing 100 embryos.
600 in horizontal position, but reversed laterally twelve to seventy-two hours after the male gametophytes were added, producing about 225 embryos.
450 rotated on the clinostat, producing more than 100 embryos.
2,000 killed and imbedded for sectioning. Approximately one-half of these were sectioned and preparations of at least 200 embryos in various stages were critically examined.

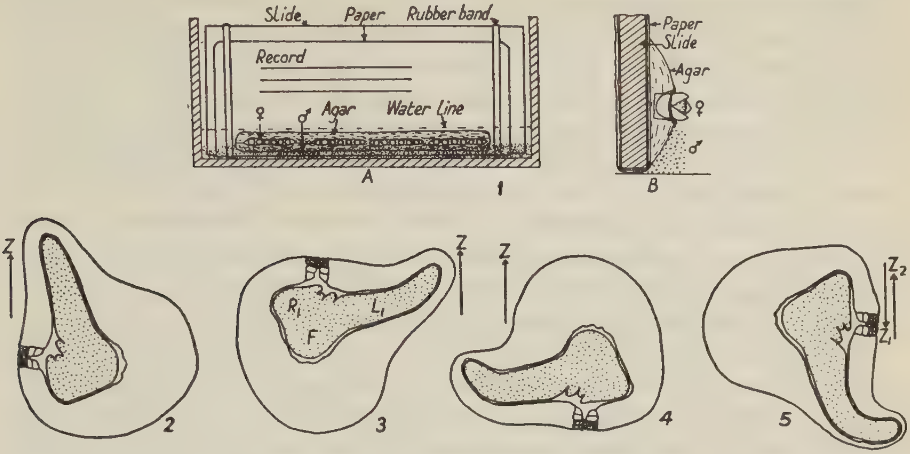
Many thousands more came under casual observation in the culture dishes and in connexion with some experimental studies of megaspore germination. Perhaps 500 or more were dissected for embryos in the living condition, and at least 100 embryos were examined in fresh or stained *in toto* mounts.

Features of the techniques described above which proved of greatest value are: (1) the fixing of megagametophytes in agar strips to be held in definite positions during embryological development and during sectioning; and (2) the removal of entire embryos from the gametophytes for staining and mounting *in toto*. The former makes it possible to section many embryos of various developmental stages in any desired plane, and the latter permits better correlation and interpretation of observations made on serial sections.

Factors Determining the Orientation of the Embryo

When the mature female gametophyte is fixed in a horizontal position so that the axis of the archegonium to be fertilized lies in a plane at right angles to the line of gravity the embryo emerges with the primary leaf pointing directly upward and the primary root directly downward (Text-fig. 2). No variation from this was observed where the fertilized archegonium was centrally located, or nearly so, in the apex of the gametophyte.

When the gametophyte is orientated with its apex upward and with the axis of a centrally located archegonium approximately in the line of gravitational force, the embryo emerges in a position at right angles to that described in the preceding paragraph; that is, so a line passing through the apices of the primary root and leaf is approximately at right angles to the line of gravity (Text-fig. 3). Relative to the archegonial axis, however, the shape and position of the embryo is the same as though the prothallium was horizontal. As the leaf apex emerges it immediately bends upward and the root bends downward; and as the young sporophyte continues to grow it straightens itself out so that all trace of its original horizontal position is finally lost.



TEXT-FIGS. 1–5. Fig. 1. *A*, diagrammatic illustration of the method of orienting megagametophytes in fixed positions. *B*, an enlarged portion of a vertical cross-section of the same 'set-up'. Figs. 2–5. Positional responses of embryos in prothallia kept in the positions indicated. The arrows show the direction of the zenith (Z) during development. In Fig. 5 the prothallus was rotated through 180° twenty-four hours after opportunity for fertilization; Z_1 indicates the position before inversion, Z_2 after inversion. R_1, primary root; L_1, primary leaf; *F*, foot.

When a prothallium is orientated with its apex downward the embryo assumes identically the same position relative to the prothallium as in the case of either the horizontal or the vertical prothallia just described, but inverted with reference to gravity (Text-fig. 4). Upon emergence, of course, the primary leaf bends upward around the basal portion of the gametophyte and the primary root bends downward, and subsequent growth eventually brings the axis of the young sporophyte into a position identical with that of the sporophytes which develop from prothallia in other positions.

When the megagametophytes are constantly rotated on a horizontal clinostat from the time the males are added until the primary leaves appear, there is no variation in position of the embryos relative to the prothallia alone from that observed in prothallia kept stationary during embryonic development.

Relative to gravity, though, there is striking contrast between the regularly vertical positions of embryos emerging from horizontal prothallia and the haphazard positions of those emerging from prothallia which have been kept in rotation on the clinostat.

These experiments indicate that either the basal location of food materials in the female prothallium, or possibly some fixed hereditary tendency, is the primary factor having to do with orientation of the embryo. Because of the radial symmetry of prothallium and archegonium, however, this factor acts as a 'single line of force' and permits other factors to come into play at various angles to this line. Here gravity becomes effective and serves as a stimulus toward vertical direction of the primary leaf-root axis; and response to this stimulus is very pronounced. Experiments in this connexion showed that one can predetermine the geographical direction of emergence of the first leaf by carefully orienting the prothallium so that the axis of the archegonium to be fertilized tilts slightly in the opposite direction.

Obviously enough, an advanced embryo cannot turn freely on the axis of its mother archegonium, and the question arises as to whether such rotation can occur even in the early more or less spherical stages. To determine this point, female gametophytes were placed on slides in horizontal position for fertilization and then rotated through 180° at given times after the male gametophytes had been added. Subsequent observations revealed that all embryos which were emerging on the sixth or seventh days from gametophytes which had been inverted in this manner thirty-six hours after the males were added to the cultures had their primary leaves emerging downward but beginning to bend toward the vertical (Text-fig. 5), while those which appeared on the eighth or ninth days had their leaves emerging directly upward as in Text-fig. 2. Pre-inversion periods of less than thirty-six hours gave decreasing proportions of inverted embryos down to eighteen hours. No embryos were inverted by pre-inversion periods of less than eighteen hours. This indicates that there is a short period beginning some eighteen hours after fertilization during which the embryo can respond to the stimulus of gravity, and that after this period there can be no change in the regular course of developmental orientation until after bending of leaf and root occurs.

Material killed and sectioned at intervals of six hours, beginning eighteen hours after the microgametophytes were added to the culture, revealed no recognizable embryos at eighteen hours or less, several two-celled embryos at twenty-four hours, and several four-celled embryos in addition to many two-celled embryos at thirty-six hours. These observations, considered in the light of those above, lead to the conclusion that the period when the embryo can respond in its position to the gravitational stimulus must correspond roughly to the time when the zygote undergoes its first mitosis. Further support for this conclusion will be brought out in connexion with the detailed account of early stages of embryonic development.

Moreover, the material killed and sectioned after seventy-two hours or more of embryonic development in vertical, horizontal, and inverted positions revealed in every instance an elongation of the embryo toward the centre of the prothallium followed by growth curvature and shift until the embryo came to lie in such position that its long axis was approximately perpendicular to the longitudinal axis of the prothallium and roughly parallel to the apical surface, as previously reported by the writer (La Motte, 1933). Hence growth curvature and shift in orientation are not responses to the stimulus of gravity, but are constant features of embryonic development regardless of positions of the megagametophytes.

Gross Morphology of the Developing Embryo

An interesting developmental series was obtained by dissecting embryos from the gametophytes and examining them in the living condition. These specimens were from cultures kept in the laboratory where temperatures usually ranged from 21 to 26° C.

The youngest embryos obtained by dissection were not more than forty-eight hours old. They varied in shape from almost spherical to ovoid and showed a multicellular condition estimated at twelve to twenty cells. No ligule initial was evident, and no dermatogen could be distinguished.

At seventy-two to seventy-six hours after the first opportunity for fertilization the largest embryos were definitely elongated and showed considerable increase in all dimensions. It was evident, however, that one side of each embryo was enlarging more rapidly than the opposite side so that the former was bulging noticeably and the latter becoming straightened. On this straightened side the ligule initial was often distinguishable but was not protruding appreciably beyond the surrounding cells. The dermatogen had also become quite definite. Most of the three-day embryos were somewhat intermediate between the stages shown in Text-figs. 9 and 10.

By the end of the fourth day the embryos were becoming quite advanced in development. The ligule of each was becoming definitely sunken by ventral invagination of the embryo, the leaf apex was beginning to extend itself, and the foot was becoming well rounded out. Curvature of the embryo was obvious at this time by reason of the concave nature of the ligular or ventral side and the convex condition of the opposite or dorsal side. Lateral enlargement was greatest in the region just below the base of the ligule.

During the fifth day the most noticeable enlargement occurred in the primordium of the primary leaf. Other parts enlarged, of course, but they maintained approximately the same proportions to each other.

On the sixth day chlorophyll made its appearance in the apical region of the primary leaf, and this leaf was then sufficiently elongated to bulge the apical tissue of the prothallium. Presence of embryos in the undissected gametophytes could be determined by observing the protrusions of gametophytic tissue and the points of green within the protrusions. Most rapid growth was again observable in the leaf region of the embryo. The primary root of the embryo was usually making its appearance at this time in the form of a noticeable bulge on the end of the embryo opposite the apex of the primary leaf.

During the seventh day, as a rule, the primary leaf became extended sufficiently to break through the end of the gametophytic sheath, and if the mother prothallium was not in horizontal position noticeable bending of this leaf could be observed. Often, too, a bulge of gametophytic tissue could be seen marking the place of development of the primary root. There was much greater variation in the time of emergence of the root than of the leaf, and frequently the former did not make its appearance for several days or even a week after the leaf had broken through.

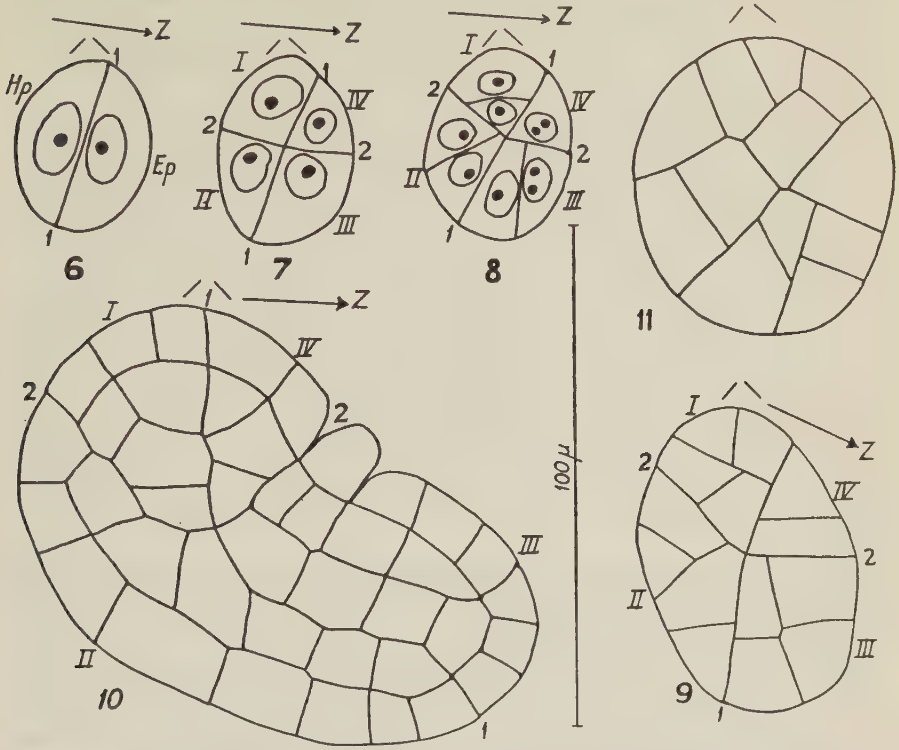
The second leaf was quite slow in its development and irregular in its time of emergence, usually appearing some two or three weeks after the first. An interesting point in this connexion is that the second leaf also protrudes through the gametophytic sheath which is formed as the first leaf develops. This fact was overlooked by the author in his previous investigation and yet it is significant in suggesting that the longitudinal axis of the rudimentary stem lies essentially in the direction of the primary leaf and root axis and not at right angles to leaf and root as might be supposed. The foot, therefore, has the appearance of being distinctly lateral in position relative to the stem axis.

Developmental progress of the embryos of the other species seemed to be very nearly the same as that of the embryos of *I. lithophila*. The embryos of *I. japonica* are often as much as twice as large as those of *I. lithophila* in corresponding stages (Text-figs. 12–14), and there seems to be slightly more elongation before dorsiventrality becomes evident. Due to failure in controlling fertilization, the time 'schedule' of development for *I. japonica* was not definitely determined, but it is most likely a little longer than the seven-day 'schedule' of *I. lithophila*.

HISTOLOGICAL DETAILS

Since the female gametophytes which provided embryos for this study were fixed in position during embryonic development, two orientation lines through the archegonium concerned can be taken to establish a plane of reference. These lines are: (1) the longitudinal axis of the archegonium, and (2) the line of gravitational force intercepting the archegonial axis at the centre of the venter. The plane which includes these two intersecting lines happens to be almost ideally located to serve as a plane of reference and, since it approximately coincides with the median plane of the developing embryo, it is herein designated as *the median plane of reference*.

The first division wall of the embryo is laid down approximately at right angles to this median plane of reference (i.e. perpendicular to the page in the drawings). Relative to the archegonial axis, however, it is characteristically so oblique in position that it forms an angle of some 20° or less with this axis (Text-fig. 6). The degree of obliqueness seems to vary somewhat with the orientation of the megagametophyte and with the position of the archegonium in the apical tissue, but rarely does it assume a position at right angles to the archegonial axis. Furthermore, the slope of this wall is such that the edge nearest the archegonial neck is always in the highest side (relative to the zenith) of the apical half of the archegonial venter as shown in Text-figs. 6, 7, 8, 9, and Pl. XXVI, Fig. 1.



TEXT-FIGS. 6–11. Early stages in embryonic development. All are of *I. lithophila*, except Fig. 11. *1–1* is the primary wall; *2–2*, the secondary; the tertiary or median wall lies in the plane of the page. The four quadrants of the embryo are designated by the numerals I, II, III, IV. Position of the archegonial apex is shown by two short converging lines; the arrow Z, indicates the direction of the zenith. Fig. 6, a 2-celled embryo 18–24 hours after fertilization. Fig. 7, a 4-celled embryo, 24–30 hours old. Fig. 8, a '48-hour' stage showing the first division walls in three of the octants. Fig. 9, a many-celled embryo at time of elongation through the base of the venter, 60–72 hours after fertilization. Fig. 10, a '4-day' stage in which curvature and shift are becoming evident. Fig. 11, an embryo of *I. echinospora* var. *Braunii* redrawn from Campbell's Fig. 22 (1891, Pl. XVI) for direct comparison with the embryos of *I. lithophila*.

The primary wall makes, therefore, a two-way positional response to gravity: (1) it takes a position at right angles to the plane of reference, which, by definition, includes the line of gravitational force passing through the centre of the zygote, and (2) it tilts in the archegonium in such a way that a peripheral point nearest the archegonial neck is in the highest portion of the apex of the venter. No exception to this was observed for any definitely determined position of the gametophyte; but observations made on sections of embryos in inverted prothallia were limited to three series and may seem insufficient for establishing the exact behaviour in up-side-down gametophytes. Dozens of observations, however, contributed to determination of the described condition in horizontal and upright prothallia.

After the primary wall is formed, the two resulting cells divide, as a rule simultaneously and in the same plane, and together lay down the secondary or 'quadrant' wall, thus forming a four-celled embryo with all four nuclei lying approximately on the median plane of reference. This secondary or 'quadrant' wall does not cut the embryo into cells of equal size. The two nearest the archegonial neck are typically smaller than those in the base of the venter, and of the two in the base of the venter the cell nearest the zenith, when the archegonium is horizontal, is typically the larger. Hence each cell of the four may at this time, on the basis of its size and position, receive a definite designation. So each is given in turn one of the numbers I, II, III, or IV, beginning with the cell at the archegonial neck as No. I, designating its sister cell on the same side of the primary wall as No. II, the other juxtaposed to the latter as No. III, and the smaller remaining one as No. IV (Text-fig. 7).

The four cells of the embryo next divide simultaneously, or nearly so, and form the median wall approximately in the median plane of reference. The embryo is thus divided into octants.

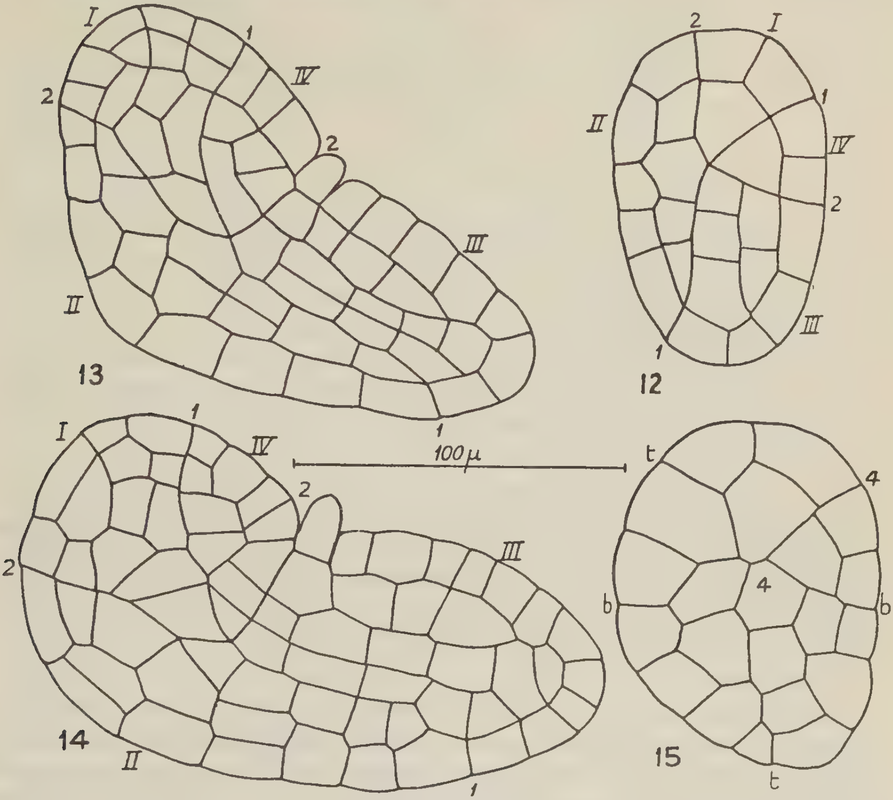
Cell divisions now cease to be simultaneous. The cells in quadrants II and III, the quadrants in the case of the venter, begin to divide more rapidly than the others and soon the embryo becomes obviously longer in the direction of the centre of the prothallium (Text-figs. 9 and 12). And since a disproportionate share of this elongation is due to growth in quadrant II, the primary and secondary walls become bent to a greater or lesser degree. Hence, parts of the two may even come to lie in approximately the same plane and together may be mistaken for either the primary or the secondary wall (Text-fig. 12). This fact probably accounts for the misinterpretations of earlier investigators and will be referred to later.

As growth proceeds in quadrant III, a cell is soon cut off in one of the octants of this quadrant next to the secondary wall of the embryo to become the ligule, thus positively identifying this portion of the embryo as the 'Anlage' of the primary leaf. Moreover, curvature of the embryo becomes evident at this time, due chiefly to the relatively slow development of quadrant IV (Text-figs. 10, 13, and 14).

Quadrant I, which at first has the position and often some of the characteristics of a vestigial suspensor (Pl. XXVI, Fig. 1), undergoes considerable enlargement and soon gives rise to the primordium of the primary root.

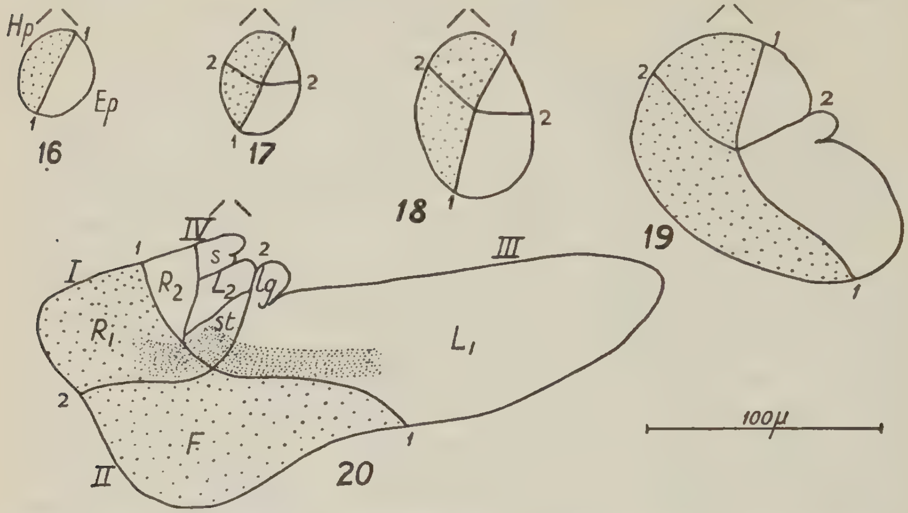
This enlargement, as well as some enlargement in quadrant IV, and the disintegration of gametophytic tissue by the foot, provide for enough shift in the position of the embryo to bring it into its final horizontal position for emergence of leaf and root (Text-figs. 16–20).

When rapid cell-division finally gets under way in quadrant IV the initial cells of the second leaf can soon easily be recognized. Then just basal to the abaxial side of this second leaf, and still within quadrant IV, the primordium of the second root becomes organized (Text-fig. 20 and Pl. XXVI, Fig. 2). The procambial strand of the first leaf unites with that of the first root just below the base of the second leaf primordium and as the strands of the second leaf and the second root become differentiated an X-shaped vascular axis is formed as described by Baldwin (1933) for *I. Engelmanni.*



TEXT-FIGS. 12–15. Figs. 12, 13, and 14, three stages in the embryogeny of *I. japonica*; Fig. 15, an early embryo of *I. lacustris* redrawn from Kienitz-Gerloff's Fig. 11 (1881, Taf. VIII): *b–b*, 'Basalwand'; *t–t*, 'Transversalwand'; *4–4*, the first wall formed in the octant. Other designations are the same as those in Figs. 5–10.

Development of the young sporophyte was not followed further in the present investigation, but from Baldwin's clear-cut figures it seems probable that the entire axis of the mature plant, including the 'rhizomorph', has its ultimate origin in quadrant IV, the quadrant longest delayed in its early development. On this basis, quadrants I, II, and III, giving rise respectively to the first root, foot, and first leaf, may be considered temporary and quadrant IV permanent.



Text-figs. 16–20. Diagrams of successive stages in the development of the embryo of *I. lithophila* showing growth curvature and shift in orientation. Fig. 20 shows, also, the most probable relation of parts to the embryo quadrants. Hypobasal portions are coarsely shaded; the epibasal are clear. The finer shading represents early differentiation of the procambium. (Cf. Fig. 22.) L_1, first leaf; L_2, second leaf; R_1, first root; R_2, second root; *F*, foot; *S*, sheath of the primary leaf; *St*, stem 'Anlage'; *lg*, ligule.

Discussion

Campbell's (1891) figures show certain stages in the embryogeny of *I. echinospora* var. *Braunii* to be almost identical with corresponding stages in the embryogeny of *I. lithophila*. To emphasize this, the writer has copied in reverse the cellular outlines of his Fig. 22 and enlarged the figure enough to give it approximately the same scale of magnification as the drawings of *I. lithophila* embryos. A comparison of this reversed drawing (Text-fig. 11) with Text-figs. 8 and 9, which represent slightly earlier and slightly later stages in *I. lithophila*, reveals close similarity of cell arrangement in the early embryos of the two species.

Similar comparisons of later stages also show very close resemblance between the embryos of the two species. Unfortunately, however, there is a gap in Campbell's series. His next stages in median section older than that discussed above are shown in his Figs. 27 and 28. Both of these are equally or more advanced than the *I. lithophila* embryo represented in Text-fig. 10 of this paper and are too advanced to present an obvious relation of parts of the embryo to the quadrants shown in his Fig. 22. (In comparing

Campbell's figures with figures in this paper one should interchange the letters *L* and *R* of his Fig. 28.) The gap in Campbell's series undoubtedly accounts for his misinterpretation of the origin of parts of the later embryo as related to the quadrants of the early embryo. Although his statements regarding his interpretation of the origin of parts are unqualified, he does say, with reference to the regularity of early quadrant divisions, that 'on account of the great difficulty of making successful sections of the very young embryos, it was not possible to get a sufficient number of satisfactory preparations to determine positively or for how long this regularity of growth persists (1891, p. 243).

At any rate, there is nothing in Campbell's figures of the early embryo suggesting an exoscopic embryogeny.

Kienitz-Gerloff's figures (1881, Tafel VIII) show the early embryogeny of *I. lacustris* in detail and represent the developmental stages with great accuracy. In no case, however, does Kienitz-Gerloff indicate the orientation of the embryo in the prothallium except when referring to the probable sequence of the first three walls of the embryo. All three walls had formed in the youngest embryos he observed, but 'bei der zur Richtung des Archegonienhalses senkrechten Form jugendlicher Embryonen liegt jedoch die Vermuthung nahe, dass die Basalwand zuerst auftritt' (p. 787). From this statement it seems that the embryo must have been observed to elongate horizontally with reference to the apical surface of the prothallus. But Hofmeister (1862, p. 344) in his description of the early embryo of the same species says, 'The rudiment of the embryo when four-celled grows toward the middle point of the spherical prothallium by repeated division of the cells turned away from the canal of the archegonium.' So there is lack of agreement on this point in spite of the fact that both used *I. lacustris* material.

It is unfortunate that Kienitz-Gerloff did not describe his methods of investigation. His figures appear to represent optical sections of entire embryos rather than microtome sections. If this supposition be correct, he probably experienced great difficulty in determining the orientation of early embryos, and he may, in that event, have been unduly influenced by the obvious positions of advanced embryos.

But the histological details shown by Kienitz-Gerloff agree very closely with those observed in the present study. To emphasize this agreement and to facilitate direct comparison of his interpretations with those of the writer, his Fig. 11 (Tafel VIII) has been copied to a scale of magnification approximately that of the *I. japonica* drawings and appears as Text-fig. 15 of this paper.

A careful comparison of this figure with Text-figs. 9 and 12 makes the differences of interpretation clear. In the first place his line *b–b* does not represent the primary wall as he supposed from observation of octant stages. This wall *b–b* might better have been considered to be the secondary wall and designated *t–t*, but even that designation would not have been fully correct. The most important difference of interpretation involves his line *t–t* which represents what he considered to be the secondary wall. Comparison of his drawing with Text-fig. 12 and other figures shows that the present writer considers this line to be representative of one portion of the primary wall plus one portion of the secondary wall. If, therefore, the *t* on the upper left of Kienitz-Gerloff's figure, as orientated by the writer, were moved over to the position of the 4 on the upper right and the *b* on the left moved up to the original *t* position there would be exact agreement, provided that *t–t* then represents the wall which separates the epibasal portion of the embryo from the hypobasal.

The errors made by Kienitz-Gerloff are easily accounted for. More rapid growth in the foot quadrant (quadrant II) than in any one of the others causes bending of the primary and secondary walls to such an extent that portions of the two come to lie almost in the same plane and may easily be mistaken for a single continuous wall at any time after the octant stage is reached (cf. Text-figs. 9, 10, and 12). This error was made during the earlier part of the present investigation but was corrected when the earliest division stages were finally obtained and studied carefully in the light of their orientation relative to the direction of gravitational force. In view of the fact that the primary wall is always inclined toward the highest point in the apical half of the archegonial venter and because the cell nearest the neck of the archegonium remains recognizable through several divisions, the deceptive bending of primary and secondary walls becomes obvious. Had Kienitz-Gerloff obtained the two- and four-celled stages, however, he would most likely have made correct interpretations, because his account of later stages is unquestionably thorough and accurate in histological detail.

A comparison of the embryogeny of Isoetes with that of other pteridophytes whose embryogenies are known reveals that it is distinctly different in one or more fundamentals from that of any other. In many respects it compares quite favourably with that of the leptosporangiates. In both of these groups the basal wall is the first one formed; leaf, stem, root, and foot quadrants are characteristically definite; the first leaf appears far in advance of the second and is distinctly quadrant in origin; and there is no hypocotyl. Furthermore, Leitgeb's (1878) experimental work with Marsilea shows a similar response of the zygote to the direction of gravity, except that the plane of the primary wall includes the axis of the archegonium.

Differences of possible importance are found in the position of the primary wall, in the juxtaposition of quadrants, and in the lack of definite apical cell growth in Isoetes. In juxtaposition the quadrants of the leptosporangiates are in root-foot-stem-leaf sequence when taken in order around the periphery, while those of Isoetes are in root-foot-leaf-stem sequence.

Whether this difference in juxtaposition is a fundamental one may be evident from a comparison of the embryos of Isoetes and Marsilea. In Isoetes the primary leaf of the embryo develops with its ventral surface outward and opposite the foot, and with the stem apex between the primary leaf and primary root; in Marsilea the ventral surface of the primary leaf is turned inward and towards the foot. The epibasal portion of one embryo is thus reversed with respect to the epibasal portion of the other, and since the epibasal portion is presumably more conservative than the hypobasal there is little likelihood that one of these types has been derived from the other. It would seem, then, that these two types represent independent development to the same level of definite segmentation from a more generalized indefinite type. If this be the case, it follows that both of these types may be more closely related to certain less definite and more primitive types than they are to each other.

Comparison of the embryogeny of Isoetes with that of Lycopodium or of Selaginella shows no close similarity. In both the latter forms the primary wall of the embryo characteristically separates the zygote into suspensor and embryonic cell, and the fourth wall instead of the first becomes the 'basal wall'. Furthermore, there is apparently less definite relation of embryo parts to the first cell cleavages; the stem apex and hypocotyl are the first to be distinctly differentiated; the root is usually epibasal in origin and does not involve an entire quadrant; and two 'cotyledon' initials are laid down, though one may be somewhat delayed in its development. In contrast with this the Isoetes embryo manifests a definite relation of parts to earliest cleavages; the stem apex is long delayed in its development and no vestige of a hypocotyl is formed; the primary root is hypobasal and quadrant in origin; and there is a single 'cotyledon' with its ontogeny distinctly quadrant in nature and definitely different from that of the second leaf.

Careful comparison of the embryogeny of Isoetes with that of the Psilotaceae or of the eusporangiate ferns reveals little in the indefinite embryogeny of the latter suggestive of the definite origin and orientation of parts as seen in Isoetes, and hence little to indicate close affinity between them and the Isoetaceae.

Support for such an isolated position of the Isoetaceae as the embryogeny suggests is found in the many unique anatomical features of the mature sporophyte. The very short axis with its sunken apical meristems and anomalous secondary growth; the origin of roots from the remarkable basal portion or 'rhizomorph'; the invaginated foliar position of the large, indehiscent sporangia subdivided by trabeculae; the relatively large leaves, each having a single unbranched collateral vascular strand and large lacunae divided by transverse diaphragms; and the eccentric position of the monarch stele of the root—all of these features have no known counterparts among other vascular cryptograms of the present day, and yet all are strikingly uniform throughout the family.

Among features possessed in common with certain other lycopsid forms, the outstanding ones are: the presence of a ligule and the attainment of heterospory, possibly linking the Isoetaceae with the Selaginellaceae; regular dichotomy of roots and occasional dichotomy of stem found as the usual condition in various lycopods; and the protostelic vascular structure common to many of the more primitive forms.

The most apparent evidences of affinity between the Isoetaceae and the Selaginellaceae are found in the structure and development of spores and gametophytes. The megaspores and megagametophytes, especially, show close resemblance—so strikingly close that an independent origin is almost inconceivable. The microspores and microgametophytes show less resemblance in several respects and are strikingly different in the number and characteristics of the sperms produced (Dracinschi, 1933; Yuasa, 1933).

The serological studies made by Conradi (1926) revealed little in common between Isoetes and either the Selaginellaceae or the Lycopodiaceae and seemed to indicate a closer relationship with the Psilotaceae.

It seems, therefore, that the sum total of present information obtained from the study of living forms favours the view that the Isoetaceae are far removed phylogenetically from all other groups and that close affinities are to be found, if at all, only among fossil forms.

The Isoetaceae have no well-established fossil record, but an Early Cretaceous form, Nathorstiana, recently described in considerable detail by Maegdefrau (1932), seems to be very closely related to Isoetes. This plant, with a stem about 8 cm. in length and some 3 cm. in diameter, possessed a basal rhizophore of two to four lobes, leaves very similar to those of Isoetes arranged in a crowded spiral, dichotomous roots with an apparently eccentric stele arranged as on the Isoetes rhizomorph, and other characteristics indicating close relationship to our present-day representative of the family. Unfortunately, nothing is known about the spores.

An older form from the Triassic 'Buntsandstein', Pleuromeia, is better known. It was described rather fully by Solms-Laubach in 1899 and by Potonié in 1904, but has attacted greatest attention in more recent years and has lately been treated extensively by Maegdefrau (1931) and Hirmer (1933). Hirmer's restoration (Abb. 1) is of special interest.

The axis of the plant, always unbranched, reached a height of 2 metres and a diameter of about 9 cm. The enlarged base, or rhizophore, was usually two- rarely three-lobed, and each lobe forked once dichotomously. On this rhizophore the roots were borne thickly over the surface, leaving elliptical scars which show each root to have possessed an eccentric vascular strand. The vascular axis of the plant was protostelic and probably without secondary growth, so that the increased diameter of the stem must have been due to an anomalous type of cambial activity similar to that in Isoetes. The slender leaves with broad bases each possessed a prominent double, but unbranched, mid-vein, and were closely arranged in spirals. Ligules were present.

The rather structureless sporophylls, which were probably lacking in vascular tissue, were arranged in a single terminal strobilus some 20 cm. in length and about 2·7 cm. in diameter. They were broad, rounded processes scarcely longer than the sporangia. The plant was heterosporous with spores closely similar in size and other characteristics to those of Isoetes (Fitting, 1907; Maegdefrau, 1931). But, strangely enough, the sporangia appear to have been abaxial and, since many morphologists and palaeobotanists consider the position of the sporangium to be most significant in determining affinities, there has been much hesitancy in assuming any real relationship between the Pleuromeiaceae and the Isoetaceae.

Williamson (1887, p. 41) was perhaps the first to emphasize some of the striking resemblances between Isoetes and some of the lepidodendrids when he said:

> 'In two respects the affinities of the Stigmarian rootlets with those of Isoetes are remarkable. In both organisms these rootlets are given off from the lower part of a downward prolongation of a caulome, which prolongation never develops leaves; the rootlets, therefore, are produced upon an axis which grew in the opposite direction to that in which the leaf-bearing parts of the stem grew. In addition to this, the rootlets of Isoetes and of Stigmaria agree in the circumstance that, in both, they are converted, during life, into fistular cylinders, owing to the disappearance or non-development of the delicate parenchyma, which ought to occupy the space between the outer cortical layer and the investing sheath of the central vascular bundle.'

And in 1897 Bertrand, Cornaille, and Hovelacque wrote (p. 243):

> 'Nous conclurons en disant: que nous croyons avoir montré . . . que les peculiarités de l'organisation du stipe et de la fronde des *Isoetes* qui les isolent des plantes actuelles sont précisément celles qui permettent de reconnaître leurs affinités et de les rattacher intimement aux Lépidodendrons. *Isoetes* est le dernier représentant de ce type végétal.'

Potonié (1902, p. 753) also recognized the many similarities, and in conclusion said:

> 'Insbesondere sei darauf aufmerksam gemacht, dass von den echten Stigmarian bis zu den *Isoetes*-Stämmchen in morphologischer Hinsicht in den Stigmariopsen und in *Pleuromeia* Übergangsbildungen zu den Isoetes-Stämmchen gegeben sind —so dass wir die Reihe erhalten: 1) *Stigmaria*, 2) *Stigmariopsis*, 3) *Pleuromeia* und 4) *Isoetes*-Stämmchen,—wie man sie sich nicht besser wünschen kann. . . . Man könnte geradezu die Isoetaceen als getauschte Sigillariaceen bezeichnen.'

Lang (1915), after a detailed investigation of the Isoetes 'stock', placed emphasis on the idea previously advanced by Fitting (1907) that a comparison of the rhizophore of Isoetes with that of Pleuromeia and the earlier forms must take into account the 'horns' of the Isoetes stele rather than the parenchymatous lobes, and on the basis of such interpretation he considered the evidences of homology to be convincing.

West and Takeda (1915), in their thorough investigation of the Isoetes 'stem' recognized the rhizophore as 'an organ sui generis', but they concluded that the presence of a distinct root-bearing organ does not necessarily indicate affinity with stigmarian forms or their derivatives. In their opinion the resemblance is merely superficial, because the arms of the stele are in the centres of the lobes in Pleuromeia and the earlier lepidodendrids but alternate with them in Isoetes. However, Lang's comparison of the stelar axis of the Isoetes rhizophore with the basal lobes of Pleuromeia seems far more convincing. The parenchymatous lobes of Isoetes are merely food-storage structures which probably have little morphological significance and should not be allowed to obscure the real resemblances.

Grenda (1926) recognized the apparent homologies, but he inclined to the belief that the Isoetaceae are very primitive in nature and represent an ancient line which has shown little advancement except to give off branches such as the Pleuromeiaceae which became extinct. But Maegdefrau (1931, p. 136) says, 'Die Annahme Grendas, dass die Isoetales schon am Ende des Devons auftreten und im Karbon "unter den dürftigsten Bedingungen" lebten, entbehrt jeglichen paläobotanischen Beweises', and for this reason he believed that the Sigillaria-Pleuromeia-Isoetes series may represent in a general way the actual descent by progressive reduction from an ancient Sigillarian stock. Then, after a detailed consideration of the Triassic genus, Nathorstiana, Maegdefrau (1932, p. 715) was convinced 'dass die phylogenetische Reihe *Sigillaria-Isoetes* zu den am besten bekannten des Pflanzenreichs gehört'.

Eames (1936) believes that the resemblance of the base of Pleuromeia to that of Isoetes is so close that there can be little doubt of the morphological similarity of the two, and after consideration of other structural similarities between these two forms he says (p. 355), 'The supposedly dorsal position of the sporangia would keep them apart, but this is probably a misinterpretation.' In another place (p. 353) he hopefully predicts that 'Some other interpretation of the anomalous position of the sporangia will doubtless be made when better preserved material is obtained'.

The present palaeontological evidences indicate, therefore, a very strong probability that Isoetes, by reason of its adaptive reduction, remains as the sole living representative of a long line going back to a larger and more complex Sigillarian or Archaeosigillarian ancestry, distinct even in the Carboniferous from the Lycopodiaceous or Selaginellaceous lines of descent.

PHYLOGENETIC CONCLUSIONS

In the light of our present knowledge of the embryological and other morphological features of the genus Isoetes, and in view of the relevant palaeontological facts now available, the safest conclusions would seem to be:

a. That the embryogeny of Isoetes is of an advanced endoscopic type probably derived from some more or less primitive type by abolition of suspensor and suppression of the stem apex.

b. That the Isoetes sporophyte has evolved from a larger, more complex microphyllous form through reduction of the stem and through development of the capacity for dormancy in maintaining its perennial habit under extremely difficult environmental conditions which greatly reduced competition from other forms.

c. That heterospory and the possession of a ligule are very ancient features probably retained from some common primitive stock by both the Selaginellaceous and the Isoetaceous lines of descent.

d. That the Isoetaceae constitute an isolated group standing well apart from all other families of present-day vascular cryptogams but very probably more closely allied with the living and fossil Lycopsida than with the Pteropsida.

e. That Isoetes is the most advanced of the present-day Lycopsida in the same sense that the 'Hydropterids' may be considered the most advanced of the present-day Filicineae.

f. That the closest affinities of the family are with the Mesozoic Pleuromeiaceae and, through them perhaps, with the Paleozoic Sigillariaceae.

g. That in taxonomic treatment the Isoetaceae deserve to be set apart as a separate order, the Isoetales, which should include no other living forms now known, but which may include the Pleuromeiaceae and possibly other palaeobotanical forms.

h. That some revision of the older systems of classification is needed as an approach to a more natural grouping of the lycopsid forms if the Isoetaceae are to be recognized as widely separated from the Selaginellaceae, Lycopodiaceae, &c., and at the same time more closely allied with them than with any of the orders of the eusporangiate or leptosporangiate pteropsids. For this reason, not to mention others, the newer system proposed by Eames (1936) seems preferable to the older systems of classification.

SUMMARY

1. A detailed study has been made of the early embryo of Isoetes under controlled conditions which would reveal the possible effect of gravity upon its form and orientation.

2. Mature megagametophytes were placed on strips of agar attached to glass slides for definite orientation during development of embryos or for rotation on the clinostat.

3. Prothallia which were kept in fixed positions during and following fertilization produced embryos with first leaf and first root in vertical position when the gametophytes were horizontal, and in horizontal position when the prothallia were in either the upright or the inverted position. The primary leaf always appeared in a direction opposite to the direction of inclination of the archegonial axis from the zenith and at right angles to that axis. It then bent upward after emergence, when necessary, in order to attain the vertical position. The root invariably appeared opposite the leaf relative to the archegonial axis.

4. When horizontal gametophytes were changed to an inverted horizontal position by rotation through 180° within eighteen to twenty-four hours after fertilization the embryos were inverted also, so that the first leaf emerged

downward and the first root upward. When this change in position of the prothallia was made less than eighteen hours after fertilization the embryos were not inverted.

5. When the gametophytes were rotated on the clinostat the embryos assumed the usual position relative to the archegonial axis but, of course, emerged haphazardly, with reference to the direction of gravitational force. None of the experimental conditions caused the embryo to vary appreciably from its advanced position in the prothallus with root and leaf approximately at right angles to the archegonial axis and with the foot turned toward the basal food supply.

6. Sectioned material revealed that the primary wall of the embryo is formed some eighteen to twenty-four hours after fertilization in an oblique position relative to the archegonial axis and with the edge nearest the neck always in that side of the apical half of the archegonium closest to the direction of the zenith. Furthermore, this wall is laid down at right angles to a plane including both a line of gravitational force and the archegonial axis.

7. Developmental responses to the stimulus of gravity are limited to the position of formation of the primary wall and to growth curvatures following emergence of leaf and root. Growth curvature and shift during the intervening stages are constant embryological features which are independent of the direction of gravitational force.

8. The primary wall serves as the basal wall by dividing the zygote into an epibasal half turned obliquely toward the base of the archegonium and an opposite hypobasal half.

9. The secondary walls divide the embryo into unequal quadrants and the median tertiary walls divide it further into octants. Of the epibasal quadrants the one most exactly in the base of the archegonium gives rise to the primary leaf, while the other very tardily gives rise to the stem. Of the hypobasal quadrants the one at the archegonial neck produces the root; the other develops into the foot. The embryo is, therefore, endoscopic and of a definite quadrant segmentation type.

10. Theoretical considerations based on our present embryological, anatomical, and paleontological knowledge suggests the recognition of an Isoetales order within the division 'Lycopsida' co-ordinate with the Lycopodiales, Lepidodendrales, and others of similar rank.

The writer wishes to express his gratitude to Professor John T. Buchholz for many valuable suggestions and helpful criticisms.

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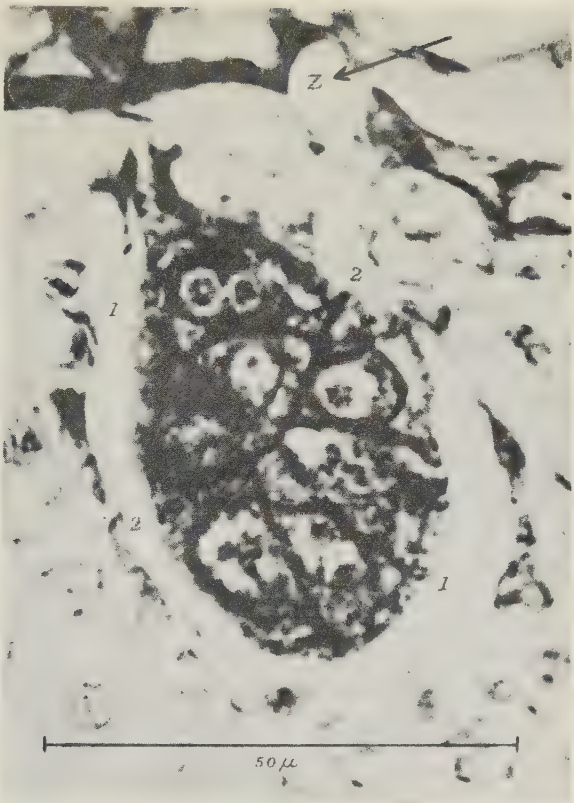
EXPLANATION OF PLATE XXVI

Illustrating Dr. La Motte's paper on 'Morphology and Orientation of the Embryo of Isoetes'.

Fig. 1. Photomicrograph of an approximately median section of an embryo of *I. lithophila* about forty-eight hours after fertilization. *1–1*, the primary wall; *2–2*, the secondary or quadrant wall. The arrow marked Z indicates the position of the zenith.

Fig. 2. Median longitudinal section of an advanced embryo of *I. lithophila* showing relation of parts to early quadrant stages. (Cf. Text-fig. 20.)



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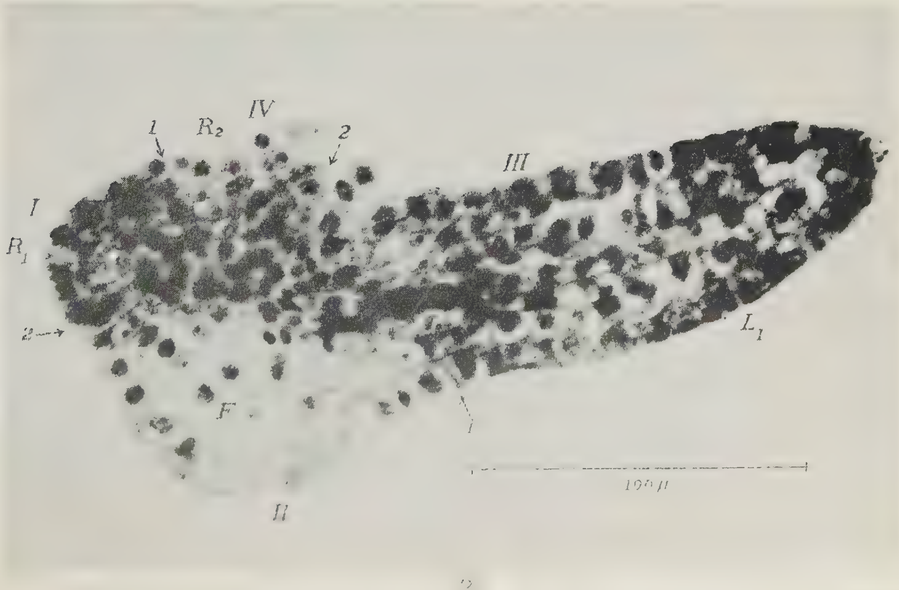

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LA MOTTE – EMBRYO OF ISOETES.

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Observations on the Distribution of Xylem-ray Tissue in Conifers

BY

M. W. BANNAN

(*Department of Botany, University of Toronto*)

With Plate XXVII and one Figure in the Text

IT is well known that rays vary greatly in height and distribution both in different parts of the xylem and from one tree to another. That these variations in some cases follow definite tendencies has been agreed upon by botanists, but in other cases varied observations have been recorded and diverse opinions expressed. For example, there is much disagreement in the literature regarding the relationships between rays and ecological conditions under which the trees are growing. Similarly, some of the observations, such as those of Fischer (1885) relative to a lack of outstanding difference between the rays in root and stem, are not in agreement with findings of the writer. In order to determine the basis of this conflict it was decided to investigate more thoroughly certain aspects of the distribution of rays and the different types of ray cells.

HEIGHT AND DISTRIBUTION OF RAYS IN STEM AND BRANCH WOOD

Much of the variation observed in the height and distribution of rays in stem and branch wood appeared to be correlated with such variants as width of the annual rings, distance from the pith, and in the case of the branch, position on the upper or lower side.

As regards the connexion between ray height and ring width, similar relationships were observed in all the conifers examined, namely, species of Araucaria, Thuja, Taxodium, Abies, Tsuga, Larix, Picea, and Pinus. At the same distance from the pith the more vigorous specimens were found to possess the higher rays. This correlation is shown graphically in the diagram, where similar trends may be seen in each of the conifers represented. The characteristic differences in average ray height in these particular species may also be noted.

The distribution of rays, as measured by the number per unit of tangential section, was also found to vary with ring width, the relationship here being of the inverse type. Rays occurred in the largest numbers in specimens with the narrower rings, where the rays were lowest. The greater number of rays generally observed in branches as compared with the stem is no doubt

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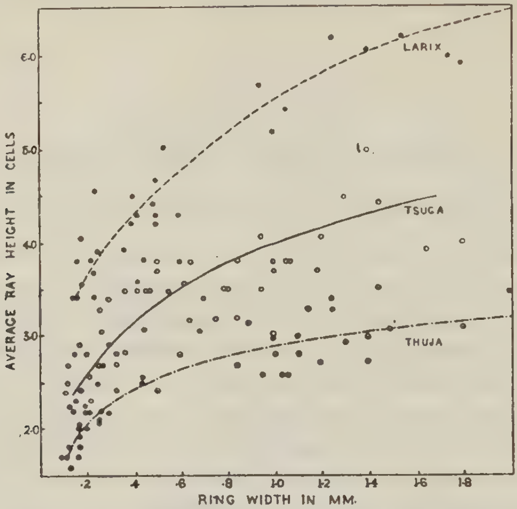
correlated with the reduced width of the rings and the associated reduction in calibre of the tracheides. These differences between branch and stem have been remarked upon by Jaccard (1915).



Average ray height and ring width at 3 mm. from the pith in stem and branch wood of *Thuja occidentalis*, *Tsuga canadensis*, and *Larix laricina*.

The distribution of rays in branches appeared in some instances to be influenced by position in the branch. In branches of *Tsuga canadensis* with a marked development of 'red' or 'compression' wood on the under side the rays in this wood were often more numerous and somewhat lower in height than the rays on the upper, 'white' wood side. Jaccard (1915) also found rays to be more numerous on the under side, but did not observe an appreciable difference in their height.

Essner (1883) found a similar number of rays on opposite sides of an eccentric system of Thuja in which the rings on one side were approximately twice the width on the other. Such a comparison, however, sheds little light on the relationship between ring width and distribution of rays, for as the graph above clearly shows, two determinations alone constitute an insufficient basis for general conclusions. Moreover, on the wider ringed side the wood is often of the 'compression' type, a factor which, in many cases at least, also has some influence on the distribution of ray tissue.

In adult stems certain tendencies in height and distribution of rays may be recognized. Essner (1883) described a gradual increase in height and decrease in number of rays outward from the pith, except in the outermost wood where there sometimes was a slight increase in number. Certain other botanists—among them Fischer (1885)—did not find this increase in the outer rings. Both types of distribution, however, have been observed by the writer, the differences apparently being correlated with age of the tree and the width of the rings. In the Abietineae which were studied for this feature it was found that in the younger trees, where the annual rings were of uniform width or narrowed only slightly toward the outside, the rays gradually increased in height and decreased in number outward from the pith. In older trees where the outermost rings narrowed sharply, the rays in these narrow rings became somewhat lower and increased in number, as described by Essner.

HEIGHT AND DISTRIBUTION OF RAYS IN ROOT WOOD AND COMPARISON WITH THAT IN THE STEM

A wide range of variation was found in the wood structure of roots, both in different specimens and in different parts of the same root. In some instances marked changes were noted in the wood outward from the centre of the root, as illustrated in Pl. XXVII, Fig. 12, for *Thuja occidentalis*, where the prominent development of thick-walled, late wood tracheides in the outer rings shows a decided contrast to the thin-walled type constituting the inner rings. This transition from an open type of wood in the centre to a more stem-like type on the outside was apparently occasioned by removal of the soil. Pl. XXVII, Figs. 7 and 9, illustrate similar differences in Picea, Fig. 7 showing the stem-like type on the upper side of a root and Fig. 9 the more open wood on the under side. Here again the differences appeared to be determined by the position of the root in the soil, as the stem-like wood was developed on the upper, exposed side. It was because of these relationships that roots were divided into two groups, 'buried' and 'exposed', when certain variations in structure were being discussed in a previous article (Bannan, 1934).

The change outward from the centre illustrated in Pl. XXVII, Fig. 12, was also observed in roots growing under conditions quite different from those described above, as for instance in certain of the sinker roots extending downward from the bole. Here the causal factor may have been the greater pressures exerted by the closely packed gravel and stones upon the roots as the latter increased in diameter.

In certain other roots the character of the xylem altered outward from the stem. The wood adjacent to the stem resembled stem wood (Pl. XXVII, Figs. 4 and 5), but in the distal parts was more open in type (Pl. XXVII, Fig. 6). In these cases the factors of depth or exposure apparently were not the determining variants as the roots were covered by soil throughout their length.

No doubt the wide variations in root structure are due to the interaction of a complexity of factors. The determination of relationships existing between these variations and the conditions under which the roots are growing is further complicated by the fact that the nature and degree of correlation differ greatly from conifer to conifer. This applies particularly to the distribution and structure of the rays.

In many conifers the rays in roots with the most stem-like wood (the 'exposed' type) resembled in height and distribution those in stems or branches of like size and age, whereas in roots with the more open wood (the 'buried' type) the rays were often quite dissimilar. The degree and trend of this intraspecific variation, however, differed greatly in different conifers.

The differences in ray height between typical 'buried' roots and branches of similar size and age are indicated in Table I. In such roots of Picea, Larix, and Abies the average ray height was just about double that in comparable branches, although it is noteworthy that the highest rays were not usually found in the deepest roots. Often the rays were higher in the distal parts of the laterals lying a few inches under the surface than in the sinkers two or three feet under the bole. In Pinus and Tsuga the differences between the 'buried' roots and branches were less distinct (Table I). Another condition prevailed in Thuja and Juniperus where little difference in ray height was observed between root and branch; and in Taxodium the rays were actually lower in the root than in comparable branch wood. The statement made in an earlier article (Bannan, 1934) that the rays are higher in 'buried' roots than in stems and branches of like size and age must accordingly be qualified. This statement applies to many conifers, but Taxodium constitutes a notable exception.

TABLE I

Comparison of Rays in Branch and Root Wood

Species.	Average ray height in cells at 3 mm. from protoxylem.		Percentage of ray rows containing 'ghosts'* or ray tracheides at 3 mm. from the protoxylem.	
	Branch.	Root.	Branch.	Root.
Thuja occidentalis	2·0	2·2	—	—
Juniperus (3 species)	2·0	2·0	—	—
Araucaria (2 species)	2·4	3·0	—	—
Taxodium distichum	3·2	1·8	—	—
Abies balsamea	4·2	8·7	14*	10*
Tsuga canadensis	2·8	4·4	26	13
Larix laricina	4·0	7·1	32	13
Picea (4 species)	3·8	7·1	33	5
Pinus Strobus	2·4	3·8	25	16
Pinus Banksiana	3·8	4·3	58	37

Pl. XXVII, Figs. 2, 10, and 11, illustrate the rays in 'buried' roots of Picea, where heights of forty and fifty cells are not uncommon, and Pl. XXVII, Figs. 14 and 17, the rays in 'buried' roots of Taxodium, generally one or two cells high. It is significant that the xylem in these roots of Taxodium (Pl. XXVII, Fig. 18) resembles that in similar roots of the Abietineae (Pl. XXVII, Fig. 6), the wood consisting of thin-walled tracheides with inconspicuous late wood in both, but despite this similarity in the tracheary elements the rays have deviated widely.

In the stem-like 'exposed' roots of the Abietineae the ray height usually resembled that in comparable stem wood, and was thus lower than in the 'buried' type. The opposite condition was observed in Taxodium where the rays in the exposed roots, or 'knees', were definitely higher than in the buried roots a few inches below.

Since the Taxodium material was collected in Southern Atlantic States and that of the Abietineae obtained locally it is rather difficult to compare the conditions under which the roots were growing. Such a comparison, however, can be made in the case of the native forms. Many of the roots of Thuja, Larix, and Picea were taken from adjacent trees, yet with respect to the height of the rays Thuja was quite unlike the other conifers. In these instances at least it would appear that the differences in the degree of variation between root and stem were not attributable to differences in the environment but were inherent in the genus or species.

TABLE II

Distribution of Ray Tissue in Wood of Root, Branch, and Adult Stem

Species.	No. of rays per sq. mm.			No. of ray cells per sq. mm.		
	Buried roots.	Branches.	Adult stems.	Buried roots.	Branches.	Adult stems.
Thuja occidentalis	63±9	67±19	56±8	135±20	140±30	185±30
Taxodium distichum	50±9	75±15	36±5	90±15	225±45	210±30
Abies balsamea	24±7	64±9	36±10	220±55	285±40	330±75
Tsuga canadensis	48±9	77±8	42±4	215±30	250±30	295±35
Larix laricina	26±6	70±7	36±4	205±35	305±35	315±40
Picea	37±6	76±10	42±6	255±50	290±40	295±35
Pinus Strobus	28±7	54±8	33±5	105±20	145±25	180±20

The distribution of rays, like the height, differed with the type of root and with the species. In Picea and Larix, where the rays in roots with the most open type of wood were more or less nearly double the height of those in stems or branches of similar size, the number of rays per sq. mm. was approximately half that in the branches. On the other hand, in Thuja the rays in such roots and branches were of similar height and comparatively little difference was observed in the numbers per sq. mm. Yet another condition was found in Taxodium where the rays were both lower and less numerous in 'buried' roots than in comparable branches.

Data showing these differences in distribution are presented in Table II. The number of rays or ray cells was determined at distances of 3 to 5 mm. outward from the protoxylem in the case of the roots and branches, and 10 cm. or more in the adult stems. The figures represent the average and the standard deviation; the extreme limits of variation noted in isolated instances were sometimes as much as twice the standard deviation.

As a rule there were fewer rays and fewer ray cells per unit of tangential section in the root than in branches of similar size and age. The amount of difference varied greatly from conifer to conifer with the least difference in Thuja and the greatest in Taxodium. Despite the smaller number of cells, the total ray volume in some species was possibly greater in the root than in other parts of the tree owing to the larger size of the individual cells. Pl. XXVII, Figs. 1, 2, and 3, illustrate the differences in size in branch, root, and adult stem wood of Picea, and Pl. XXVII, Figs. 13, 14, and 15, the differences in Taxodium.

Intraspecific variations in ray volume have been noted by a number of investigators, notably Hartig (1901), Geiger (1915), Forsaith (1920), Shope (1927), Harlow (1927), and Myer (1930). Some of the variations were believed to be correlated with certain ecological conditions, but in other cases such relationships were not apparent. This lack of agreement is not surprising, however, since a diversity of genera was studied. The present investigations have shown that even rather closely related genera may vary similarly in certain anatomical characters, and in other characters vary to unequal degree or perhaps follow opposite trends. For example, with respect to the height and distribution of rays, varied degrees of difference between root and stem were observed in different forms, the condition in Taxodium being quite unlike that in most Abietineae. Obviously the relationships between anatomical fluctuations and conditions of growth observed in one form cannot be considered necessarily illustrative of the relationships in others or in the group as a whole.

DISTRIBUTION OF THE VARIOUS TYPES OF RAY CELLS

The distribution of ray tracheides, 'ghosts', parenchyma, and the intergrading types of ray cells differs both within the species and from one conifer to another. Some indication of the generic differences, as determined from a total of 300 specimens of adult stem wood, is given in Table III. These figures must, of course, be regarded as tentative since somewhat different values would probably be obtained if other material and other species were utilized.

Ray tracheides are most abundant in certain Abietineae, especially Pinus; in Sequoia, Thuja, &c., they constitute only a small proportion of the ray tissue; and in the forms listed in the Table for which no figures are presented, are scarce or absent. In the case of Abies, Taxodium, and a few other genera not included in the Table, 'ghosts' occupy the position of ray tracheides and the ray tracheides may occur only rarely. It is noteworthy that the distribution of ray tracheides or 'ghosts' is quite dissimilar in some closely related forms, while between others the differences are slight. In the latter instances the distribution of these cells is clearly of little or no diagnostic value in separating genera or species.

Within each species considerable variation was found in the distribution of the different types of ray cells. In stem and branch wood much of this variation appeared to be correlated with age, that is, distance from the pith, and width of the rings, though no doubt other undetermined factors were also involved.

A relationship between distribution of ray tracheides and age was observed in all the conifers studied. There is a circummedullary area in which ray tracheides are absent or relatively scarce, although in those conifers with numerous ray tracheides in the more distant wood this zone may be very narrow.

TABLE III

Height and Cellular Character of Rays in the Outer Wood of Old Stems

Genus.	No. of species studied.	Av. ray height in cells.	Av. percentage of ray rows containing 'ghosts'* or ray tracheides.
Agathis	2	7·2±0·7	—
Araucaria	4	7·6±1·6	—
Dacrydium	3	4·2±1·2	—
Phyllocladus	2	3·7±0·8	—
Podocarpus	3	3·8±1·3	—
Callitris	—	9·3±3·5	—
Chamaecyparis	3	5·1±0·5	2·4±2·4
Cupressus	3	5·0±0·5	0·7±0·7
Juniperus	7	3·4±1·1	0·7±0·7
Libocedrus	2	4·1±1·7	3·0±1·8
Thuja	2	3·9±1·0	2·8±1·4
Athrotaxis	3	4·3±1·0	—
Cunninghamia	1	5·0±1·2	5·2±0·8
Sequoia	2	5·3±1·2	8·5±5·4
Taxodium	1	5·8±0·6	6·0±3·0*
Abies	3	9·2±1·9	15±4*
Cedrus	2	12·0±1·6	15±5
Tsuga	1	6·8±0·9	27±4
Larix	2	8·9±1·2	24±4
Pseudotsuga	1	7·5±1·3	26±3
Picea	4	7·0±1·1	33±5
Pinus (haploxylon)	5	5·5±1·4	42±5
Pinus (diploxylon)	13	6·5±1·0	50±8

Outward from the pith in the Abietineae the proportion of ray tracheides rapidly increases, the point at which the maximum is attained sometimes varying apparently with the conditions of growth, being closer to the pith in narrow-ringed material, as in slowly growing branches, than in the more vigorous stems. Towards the outside, especially if the ring width has remained more or less constant and the average ray height has undergone a steady increase, the proportion of ray tracheides may decrease somewhat from the maximum farther inward. However, if the rings on the outside narrow markedly, the average height of the rays usually declines and the proportion of ray tracheides sometimes becomes slightly greater again. For example, in a large tree of *Pseudotsuga taxifolia* the average ray height increased from 7·1 cells at 15 cm. from the pith to 10·6 cells at 70 cm., and the proportion of ray tracheides dropped from 29 to 21 per cent. But at 105 cm. from the pith, where the rings were much narrower, the average ray height declined to 7·7 cells and the ray tracheides increased to 27 per cent.

The decrease in ray height in the narrow rings on the outside is brought about by a dying out of ray rows, particularly the tracheary rows along the ray margins. After this stoppage the rows which were formerly submarginal in position and composed of parenchyma cells often become tracheary on the assumption of the new marginal location. As a result the proportion of ray tracheides generally is not greatly reduced, and in many cases, as in the example cited above, is actually increased. At other times, however, especially where there has been a drastic reduction in ring width extending over several years, the dying out of ray tracheides takes place faster than the change from parenchymatous to tracheary cells in the former submarginal rows, with consequent reduction both in ray height and proportion of ray tracheides.

The distribution of ray tracheides in adult stem wood of the particular Taxodineae and Cupressineae examined was more variable than in the Abietineae, and relationships of the type described above were not found.

Although the distribution of ray tracheides within the species may sometimes be influenced by the same factors that modify ray height, as pointed out in the Abietineae, there is no relationship between number of ray tracheides and ray height in the conifers as a whole. A study of Table III shows clearly that the greater development of these cells in certain genera is not necessarily associated with either a general increase or decrease in ray height.

The distribution of ray tracheides in root wood varied more than in the stem. In most of the conifers there appeared to be some correlation between the distribution and conditions under which the roots were growing.

In the Abietineae the fewest ray tracheides were found in roots of the 'buried' type. In such roots of Picea ray tracheides were so rare in some cases as to be almost entirely absent, and in the forty roots examined less than 5 per cent. of the ray rows contained tracheary cells (Table I). As parenchyma cells were scattered among the ray tracheides in these rows the actual proportion of ray tracheides was considerably less than the 5 per cent. listed. In branches of similar size and age the proportion of ray tracheides averaged 33 per cent. A marked difference between 'buried' roots and stems and branches was also observed in Larix, but in Tsuga and Pinus the differences were less distinct. A sufficient number of roots was examined to indicate that these differences in the degree of variation between root and branch were not entirely attributable to differences in the environment, but were in part deep seated in the genus or species.

When roots were above ground ray tracheides were generally more numerous than in the roots underneath and sometimes approached the proportions attained in stem wood.

In Thuja and Juniperus, as in the Abietineae, fewer ray tracheides were discovered in typical buried roots than in the exposed roots, stems, or branches, but owing to the smaller numbers of these cells in both root and stem the differences in distribution were often less noticeable.

The 'ghosts' in Abies, like ray tracheides in other conifers, constituted a smaller proportion of the ray tissue in 'buried' roots than in stem or branch wood (Table I). In Taxodium also the ghost-like types and ray tracheides were sometimes definitely less numerous in 'buried' roots than in branches of similar vigour, but in other cases were more abundant and made up a larger proportion of the ray tissue than in comparable branch wood. It may be recalled that in Taxodium, unlike other conifers, the rays in the 'buried' roots were lower than in branch wood. This lowness of the rays would to a certain extent account for the larger proportions of the ghost-like or tracheary cells.

SUMMARY

Much of the variation in height and distribution of rays in stem and branch wood of the conifers was found to be correlated with distance from the pith, position in the branch (upper or lower side), and width of the rings. The relationships were of similar character in all the genera studied.

The height and distribution of rays in the root varied more widely and, unlike the condition in the stem, the trend of variation differed from one conifer to another. For instance, in most of the Abietineae studied the rays in the 'buried' type of root were usually considerably higher than in 'exposed' roots, stems, and branches of like size and age; in Thuja the rays were lower with less difference between root and branch; and in Taxodium the rays were lowest in the 'buried' roots.

The relative proportions of the different types of ray cells also varied greatly, both throughout the tree and from conifer to conifer. In most of the forms studied ray tracheides were less numerous in 'buried' roots than in other parts of the tree, but in Taxodium the opposite condition was sometimes observed.

The fact that different conifers may vary similarly in certain anatomical characters, and in other characters vary to unequal degree or follow divergent trends must be appreciated when speculating upon the range of intraspecific variation and the relationships that may exist between such variations and ecological conditions.

To Professor R. B. Thomson I am indebted for many kindnesses.

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EXPLANATION OF PLATE XXVII

Illustrating Mr. Bannan's paper on 'Observations on the Distribution of Xylem-ray Tissue in Conifers'.

(All photomicrographs are × 55.)

Fig. 1. *Picea Engelmanni*, tangential section of branch wood.

Fig. 2. *Picea sitchensis*, tangential section of wood in 'buried' root at a point 4 cm. from the centre of the root and 2·5 m. from the stem.

Fig. 3. *Picea Engelmanni*, tangential section of old stem wood.

Fig. 4. *Picea sitchensis*, transverse section of root wood, outermost rings on the lower side, 1·3 cm. from the centre of the root and 0·3 m. from the stem.

Fig. 5. *Picea sitchensis*, transverse section of same root, outermost annual rings on the upper side, 4 cm. from the centre of the root and 0·3 m. from the stem.

Fig. 6. *Picea sitchensis*, transverse section of same root, 2·5 m. from the stem.

Fig. 7. *Picea Engelmanni*, radial section of root wood, outer annual rings on the upper, exposed, side, 22 cm. from the centre of the root and near the stem.

Fig. 8. *Picea Engelmanni*, radial section of same root, showing character of the wood just below the centre of the root, near the stem.

Fig. 9. *Picea Engelmanni*, radial section of same root, outermost rings on the under side, 3 cm. from the centre of the root and same distance from stem as Figs. 7 and 8.

Fig. 10. *Picea Engelmanni*, radial section of same root, 2 cm. from the centre of the root and 6 m. from the stem.

Fig. 11. *Picea Engelmanni*, radial section of root wood, 3 mm. from the centre and 8 m. from the stem.

Fig. 12. *Thuja occidentalis*, transverse section of root wood, 5 mm. from centre of root and 4 m. from stem, illustrating alteration in character of the xylem with removal of the soil.

Fig. 13. *Taxodium distichum*, tangential section of branch wood.

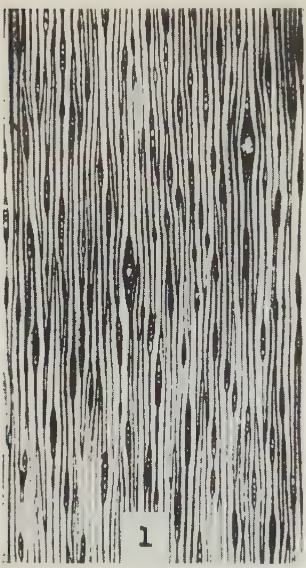
Fig. 14. *Taxodium distichum*, tangential section of root wood, 0·9 cm. from centre of the root and 20 cm. below a 'knee'.

Fig. 15. *Taxodium distichum*, tangential section of old stem wood.

Fig. 16. *Taxodium distichum*, transverse section of branch wood.

Fig. 17. *Taxodium distichum*, radial section of root wood.

Fig. 18. *Taxodium distichum*, transverse section of same root.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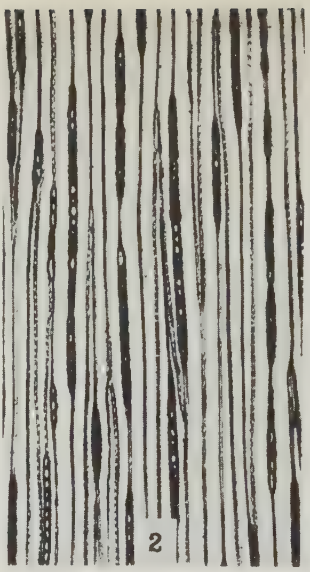
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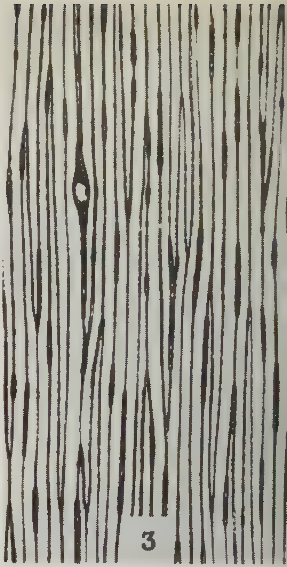
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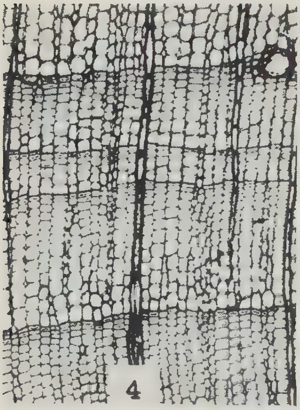
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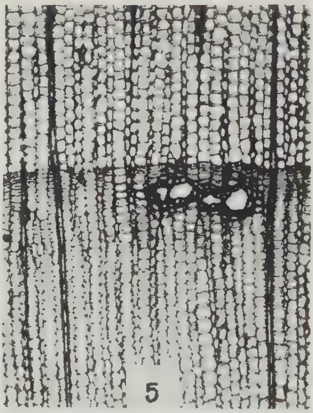
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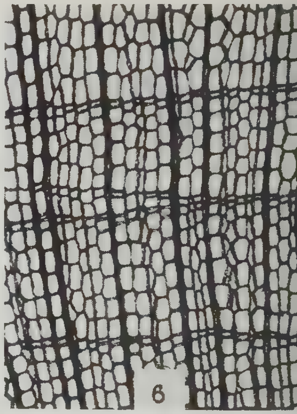
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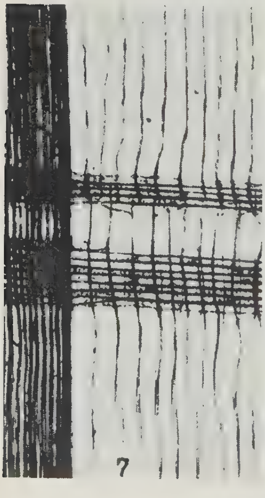
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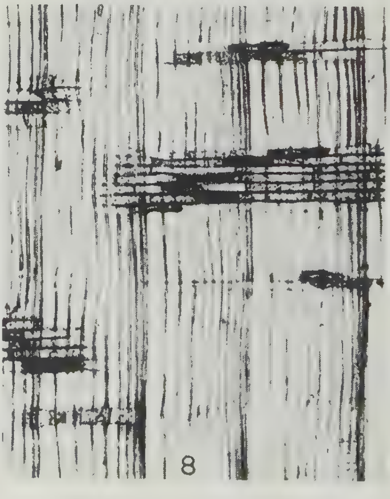
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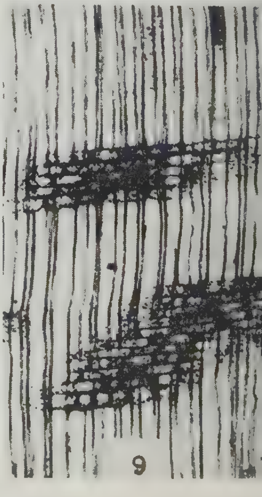
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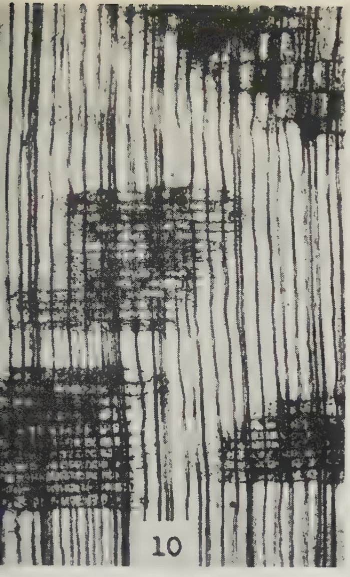
BANNAN — XYLEM RAYS IN CONIFERS.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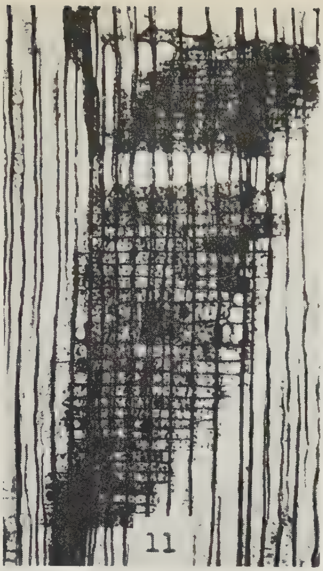
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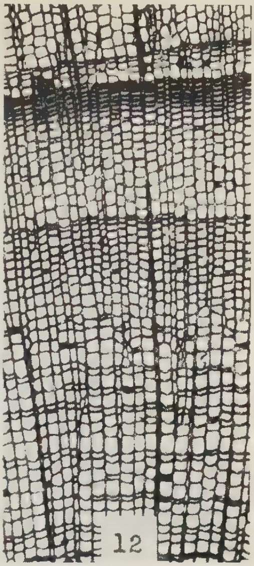
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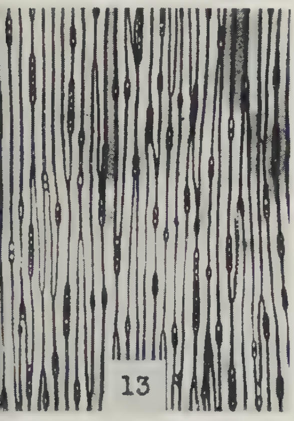
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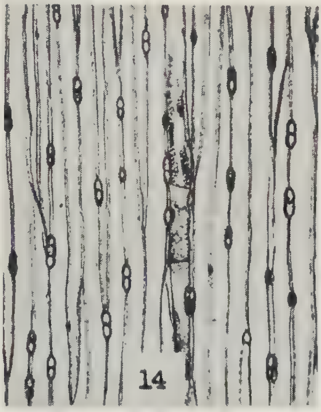
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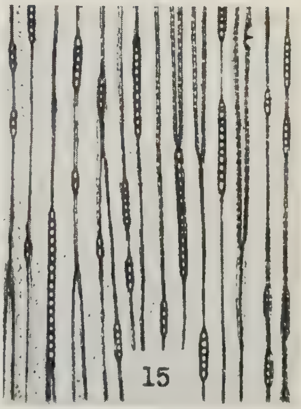
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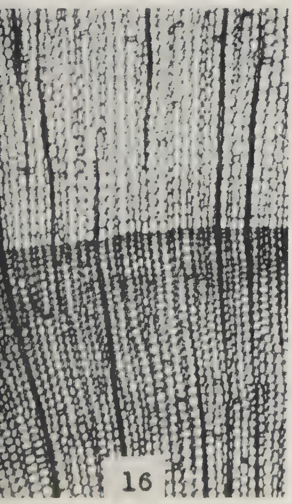
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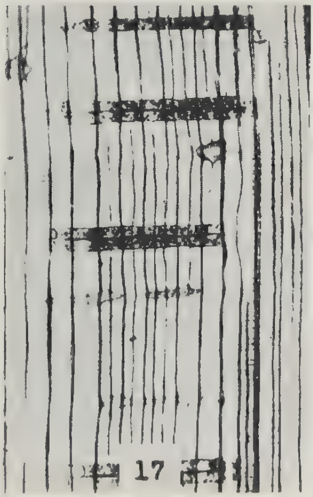
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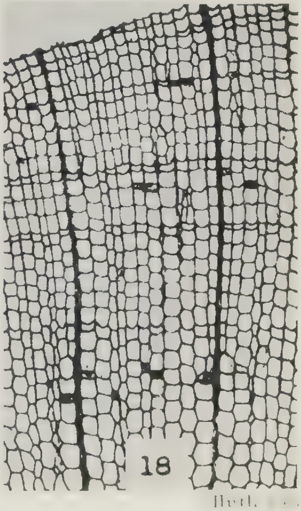
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Studies in the Physiology of Parasitism

XV. Effect of the Nutrient Medium upon the Secretion and Properties of Pectinase

BY

M. FERNANDO, Ph.D.

(*Department of Plant Physiology and Pathology, Imperial College of Science and Technology, London*)

With four Figures in the Text

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A. INTRODUCTION

THE work to be described in this paper is a continuation along certain lines of the earlier enzymological studies of this series. In comparing the pectinase enzymes of a number of parasitic fungi Chona (1932) demonstrated that they reacted differently to a number of physicochemical factors. Thus the enzymic preparations obtained from some potato-attacking fungi, *Phytophthora erythroseptica*, *Pythium* sp. and *Fusarium caeruleum* were characterized by relative insensitiveness to potato extracts, i.e. the addition of the latter to the enzymic solution had a relatively slight retarding effect on the rate of tissue hydrolysis. On the other hand, these preparations were relatively sensitive to the presence of apple juice, which strongly depressed their activity. Enzymic solutions prepared from the apple-attacking fungi *Botrytis cinerea* and *Fusarium fructigenum* showed the converse relationship. In this connexion the active principle of potato juice was shown to be certain polyvalent salts, that of apple juice to be hydrogen-ion concentration. The enzymes of the apple-attacking and potato-attacking fungi had their optima in acid and alkaline solution respectively.

Chona's results were amplified by the later work of Menon (1934), who determined the relation between pH and enzymic activity for extracts

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prepared from a number of parasitic fungi. As a rule the curves obtained for any particular organism showed the same general trend whatever the medium used or whether the enzymic solution was derived from the hyphae (endo-enzyme) or from the culture medium (exo-enzyme). Nevertheless, in one case at least the shape of the curve was markedly affected by the nature of the nutrient used.

Menon also compared the specific retarding effects of certain plant extracts on a variety of enzymic preparations. While confirming the particular results which had been obtained in the earlier work, he pointed out that the nutrient used had an important modifying effect. The experimental data were hardly sufficient to justify the framing of a rule, but they strongly suggested as a working hypothesis that the pectinase solution prepared from a culture on a particular medium tends to be relatively insensitive to constituents of that medium. The suggestion was that the enzyme adsorbed various substances from the medium and was therefore less affected by any further additions of these.

The object of the present investigation was to examine in some detail the effect of nutrient composition upon the excretion of pectinase enzyme and upon the properties of the enzymic preparations obtained. Chief attention was paid to the pH factor. While the two lines of work were carried on more or less simultaneously it will be convenient to deal with each separately and to describe first of all the effect of pH upon enzymic activity.

B. Materials and Methods

The organisms used were the following:

1. *Bacillus carotovorus* Jones, isolated from tomato.
2. *B. subtilis* (Ehrenberg) Cohn.
3. *Botrytis cinerea* Pers., isolated from lettuce.
4. *Pythium de Baryanum* Hesse, isolated from cress.

Stock cultures were maintained in tubes of potato or malt extract agar.

Enzymic solutions were obtained from *Botrytis cinerea* according to the method developed by Brown (1915), i.e. dense spore sowings were made in various media on horizontal glass plates kept in a moist atmosphere. After two days' growth at laboratory temperature, the hyphal mass was removed by centrifugalizing and the culture solution which contained the excreted enzyme was used as such in the tests.

The above method being unsatisfactory for the preparation of Pythium enzyme, the latter was prepared by squeezing out the juice from autoclaved blocks of potato tuber which had been inoculated twelve days previously with the fungus. This extract was subjected to one purification by precipitation with alcohol.

Preparations of the bacterial enzymes were obtained by adding a definite number of drops of a bacterial suspension in water to each of a series of flasks containing 20 c.c. of a liquid nutrient medium. The aim was to present the latter as a shallow layer so that good aeration would be ensured throughout the medium. From time to time a sample flask was withdrawn from the incubator and the enzymic activity of the culture fluid determined. The enzyme was used in the unpurified form and with no addition of antiseptics except where the action was prolonged on account of the low activity shown. In that case a few drops of toluene were added. After some experience it was fairly well known whether it was desirable or not to add toluene at the beginning of the tests, but for safety some experiments were run in duplicate, one set with toluene and the other without. Whenever the solution under test was so weak that the reaction was incomplete after seven hours, toluene was then added. From the purely enzymological point of view the addition of toluene is allowable, but is open to objection in so far as the idea is to study the effect of the enzyme as an agent of parasitism. In carrying out this work, therefore, the general plan was to avoid the use of antiseptics except where they were absolutely necessary.

The standard method of testing the various enzyme solutions was to determine the time required, at known temperature, for the decomposition of discs of potato tuber of standard thickness (0·5 mm. × 1·8 cm.). In each test three such discs were used, and the average taken. In any one experiment, all the test discs were cut at the same time from the medullary tissue of the same tuber, this precaution being necessary on account of the marked variation from one tuber to another. The time required for complete loss of coherence, as tested mechanically, gives an inverse measure of the enzymic activity of the solution tested.

Throughout the work parallel determinations were made of the drift of the reaction in the various cultures and of the rate of growth of the organisms.

Estimations of pH values were made with the Universal Buffer Solution prepared by British Drug Houses according to the formula prescribed by Prideaux and Ward (1924). By suitable additions of ½-normal soda, the range pH 3·1 to 11·5 can be accurately covered. The usual indicators were used.

The rate of multiplication of *B. carotovorus* was determined by the standard dilution method. This method is generally inapplicable to cultures of *B. subtilis* on account of the tendency to aggregation and scum formation. For this organism a rough estimate of amount of growth was obtained by centrifugalizing and measuring the volume of precipitate. By this means all but the finest aggregates were carried down.

Determinations of the amount of fungal growth were similarly made with the centrifuge, the wet volume of the precipitate being noted.

C. EXPERIMENTAL RESULTS

1. *The pH value as affecting enzymic activity.*

A representative set of curves illustrating the behaviour of the enzyme of *B. carotovorus* in relation to pH value is given in Fig. 1. Here pH is plotted against reaction time (R.T.), i.e. the time required for complete loss in coherence of the standard test discs of potato tissue. The preparations were derived from cultures on a variety of media and after different periods of incubation so that they showed a considerable range of activity. The optimum pH in all cases is in the neighbourhood of 8. A point of interest is that, although in the more dilute solutions the optimum is sharply defined, at the higher concentrations the curves exhibit a flat-topped optimum. This difference could be explained on the assumption that the rate of diffusion of the enzyme into the test discs of tissue is the slowest process, and as such assumes the function of a master reaction limiting the rate of maceration. The effect described has been repeatedly met with in the course of this work,

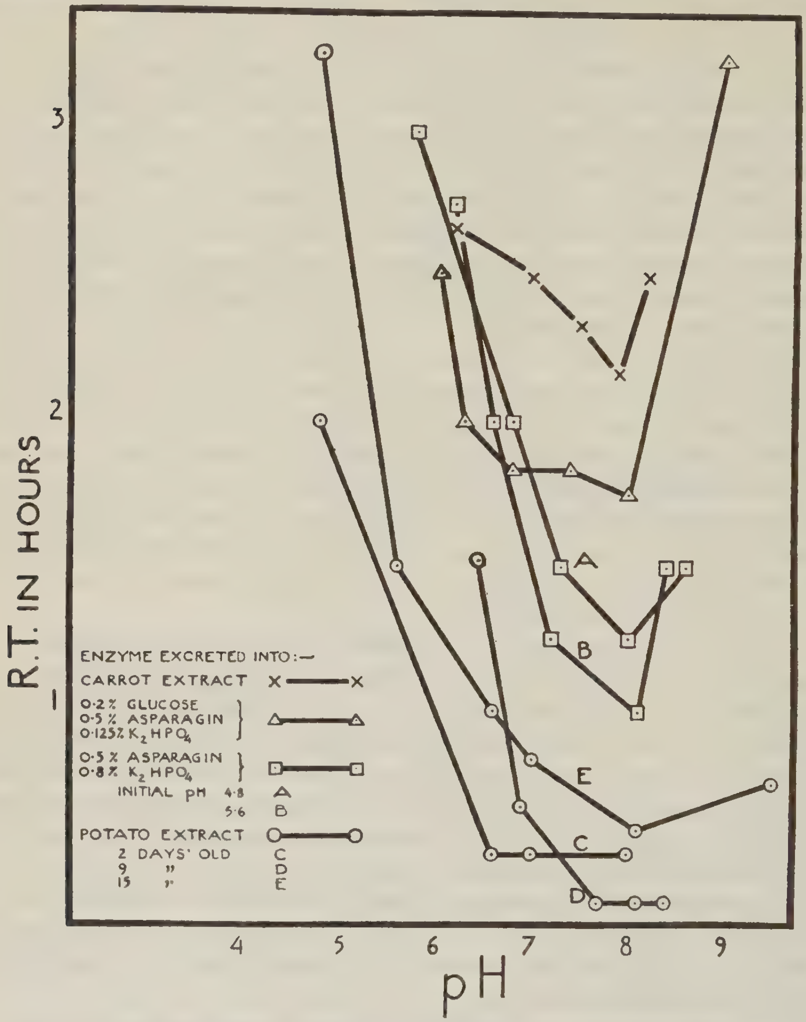

FIG. 1. Illustrating relation between pH and reaction time (R.T.) of *B. carotovorus* enzyme excreted into various media.

whence it follows that solutions of high enzymic activity should be avoided if the position of the optimum is to be accurately determined.

While the position of optimum activity in terms of pH value is more or less sharply defined and lies in the neighbourhood of pH 8, there is evidence that it tends to shift during the course of bacterial growth if the latter leads to the development of an acid reaction. Furthermore, exposure of the enzyme to acid conditions alters it in such a way that it exhibits an optimum activity at a lower pH value. These statements will now be illustrated.

In one experiment, *B. carotovorus* was grown on an apple extract (100 gm. per litre) to which the following constituents were added: 0·5 per cent. asparagin, 0·8 per cent. K_2HPO_4, and 0·075 per cent. $MgSO_4$, $7H_2O$.

The pH value moved from 7·2 to 5·5 by the third day and remained steadily at that low level for the rest of the experiment (18 days altogether). The determinations of the effect of the pH value at various intervals are reproduced in Fig. 2, in which the reciprocal of the reaction time in hours multiplied by 100 is taken as a measure of the activity of the enzyme.

From the figure it is seen that the enzyme of a 3-days-old culture had its optimum at about pH 8·5. After six days this had drifted to the region pH 6·8–7·6. In the 10-days-old culture its position was not accurately determined (6·9–8·0), but at the end of the experiment an optimum in the neighbourhood of pH 7·0 was indicated. Though more detailed examination would be required for the exact location of the various optima, the curves of Fig. 2 indicate clearly that the pH for optimal activity of the bacterial enzyme has moved in the acid direction as the cultures developed.

The same feature is brought out in a somewhat different way in the following experiment. To a basal medium consisting of 0·15 per cent. glucose, 0·5 per cent. asparagin, 0·075 per cent. magnesium sulphate was added Clark and Lubs' (1928) KH_2PO_4–NaOH buffer mixture in such proportions that the medium had initial pH values of 8·0 and 6·2. These two media were inoculated with *B. carotovorus*. Over the first five days, the pH of the former fluctuated between the limits 7·4 and 8·1; that of the latter within the limits 5·7–6·7, i.e. the one medium remained definitely alkaline and the other definitely acid throughout. Examination of the culture media after five days' incubation showed that the region of optimal activity was pH 7·7–8·3 and pH 6·8–7·8 for the enzyme secreted in alkaline and acid solution respectively. In other words, as the pH conditions prevailing in the culture solution are altered, the pH which is optimal for enzymic activity tends to move in the same direction.

The results just described could be interpreted to mean that the organism secretes an enzyme of somewhat different properties in accordance with the reaction of the medium into which the enzyme is excreted; or that the enzyme is fundamentally the same when excreted, but that its properties are modified on exposure for some time to acid solutions. The latter view appears to fit the observed results best, as the following experiments show.

In a number of experiments *B. carotovorus* was grown on the same medium which was adjusted to different initial pH values over the range 4·8–8·1. On the second or third day of incubation, the culture media were tested as to the optimal pH range of the enzyme present, and this was found to be in the neighbourhood of pH 8·0 for all the preparations. There was no indication that, in these young cultures, there was any difference in the pH response of the enzyme. The suggestion, therefore, is that the enzyme, when excreted, is fundamentally the same, and that it requires exposure for some length of time to an acid solution before its properties are definitely altered.

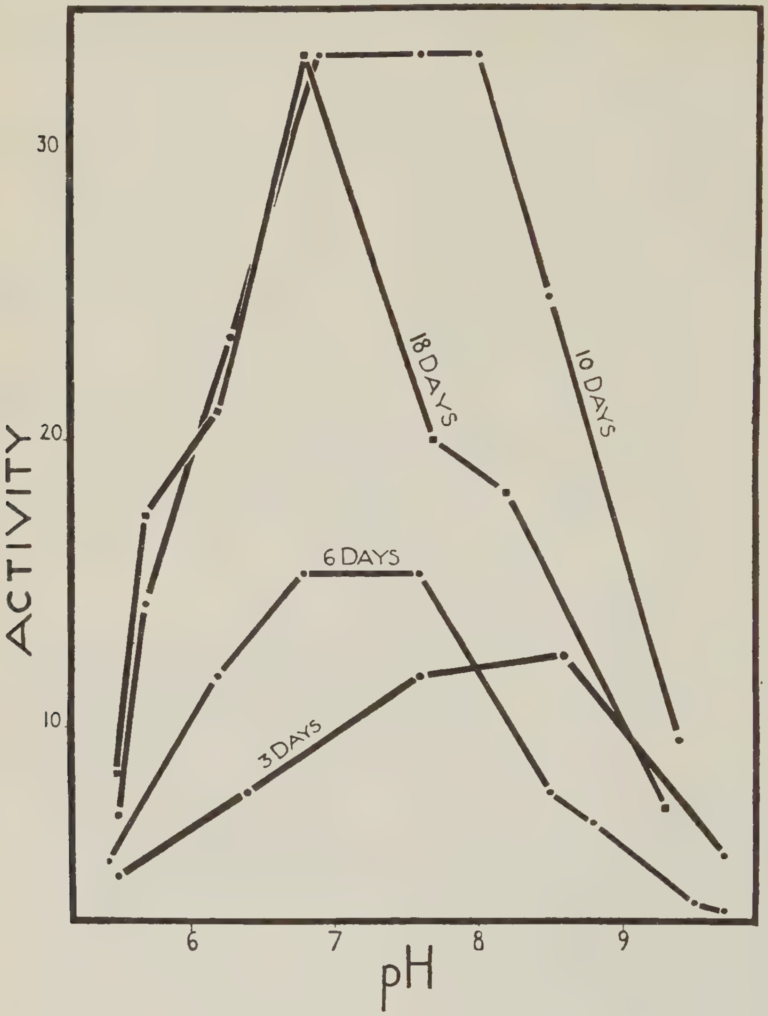

FIG. 2. Illustrating effect of pH on activity of *B. carotovorus* enzyme of various ages.

The effect of exposure of the enzyme to acid solutions was clearly shown in a series of experiments in which the enzyme was kept for varying lengths of time, in the presence of toluene, in slightly alkaline (pH 8·0) or in acid (pH 3·0) solution. The presence of toluene, in addition to preventing contamination, ensured that there was no continued secretion of enzyme throughout the period of the experiment. Fig. 3 illustrates the distinct shift of the optimal point arising from storage of the enzyme in acid solution.

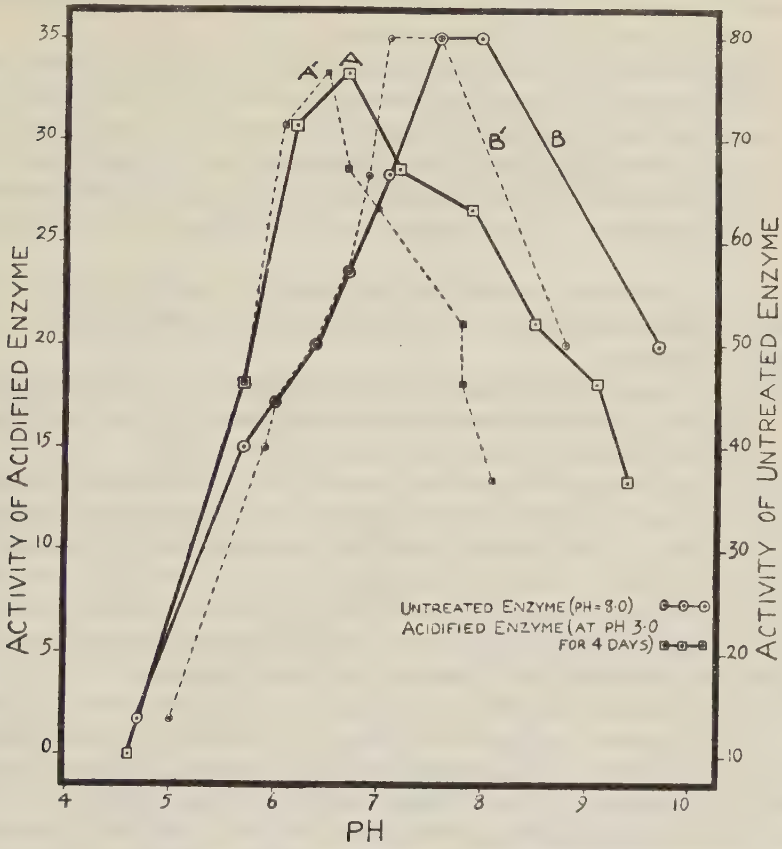

FIG. 3. Illustrating effect of exposure of *B. carotovorus* enzyme to acid conditions.

It will be observed that in the figure each curve is plotted in duplicate (*A* and A^1, *B* and B^1). The continuous curves give the activity of the enzyme against the pH of the latter before the test discs of tissue are added. Through the liberation of juice from the latter, the alkaline enzymic solutions become less alkaline and the distinctly acid solutions become less acid. The dotted curves give the enzymic activity plotted against the pH of the solutions after maceration of the discs is completed. The true curve would obviously be somewhere between the continuous and the dotted curves. This effect mentioned, which has been noted throughout the whole work, is not very great and it could be reduced by buffering the enzymic solutions or by using a larger quantity of the latter for each maceration test. The dotted curves have been omitted from Figs. 1 and 2 in order to avoid confusion.

The shift of the pH optimum, which is brought about by four days' exposure to a pH of 3·0 is from pH 7·6–8·0 to pH 6·7 or, if the dotted curves be considered, from pH 7·1–7·6 to pH 6·4.

Incidentally it will be noted that exposure to acid conditions increases the rate of inactivation of the enzyme in watery solution. This is clear on comparison of the scales used in plotting the two pairs of curves, that of the enzyme exposed to acid (B and B^1) being shown on the left, that of the enzyme kept at pH 8·0 (A and A^1) being on the right. After eight days' exposure to an acid concentration of pH 3, the activity of the enzymic solution was almost zero.

Further experiments showed that the change in position of the optimum could be demonstrated after two days' exposure to acid conditions corresponding to pH 2·0.

The most suitable hypothesis therefore appears to be that the enzyme of *B. carotovorus* has a characteristic optimum at about pH 8, but that exposure to acid conditions causes this to shift towards an acid point.

The effects of the pH value on the activity of the enzymes of *Bacillus carotovorus*, *Bacillus subtilis*, *Botrytis cinerea*, and *Pythium de Baryanum* are shown in Fig. 4. The enzymes of all the organisms except Pythium were excreted into potato extract. It has not been found possible to secure an enzyme of sufficient strength from Pythium by using potato extract. The Pythium enzyme employed was extracted from rotted autoclaved potato tissue and prepared according to the method previously described.

The curves for the activity of the enzymes of *B. carotovorus*, *B. subtilis*, and *P. de Baryanum* are very much alike, with optimal pH values distinctly on the alkaline side. The behaviour of the Botrytis enzyme is, however, different, the position of the optimum pH value being slightly on the acid side. The activity declines gradually towards the higher H-ion concentrations, but has a much steeper downward gradient on the alkaline side. The reverse holds for the enzymes of the other three organisms. Here the downward slope on the acid side is sharper than on the alkaline. In the region extending from about pH 6·7 to pH 7·5, the curve for the Botrytis enzyme shows a rapid descent, while the activities of the other enzymes exhibit a rise. At a pH value of about 9·5, at which the other enzymes have lost at most a small fraction of their activity, the activity of the Botrytis enzyme is almost nil. Again, whereas the activities of the enzymes of *B. carotovorus* and *P. de Baryanum* have fallen nearly to zero long before a pH value of 3 is reached, the Botrytis enzyme still retains at pH 3 a considerable proportion of its activity.

Brown (1915) studied the effect of the H-ion concentration on the activity of the enzyme of *B. cinerea*, but he represented his results in terms of titrable acidity and not of pH values. He found that the optimum reaction for the activity of the Botrytis enzyme was a little on the acid side of neutrality, with a sharp gradient towards the alkaline side. The curve for Botrytis in Fig. 4 resembles the curve that Brown would have obtained if he had expressed his results in terms of pH values. Menon (l.c.), on the other hand, observed that the curve for the activity of the Botrytis enzyme rose continuously till a pH value below 3 was reached. Thus while there may be some disagreement

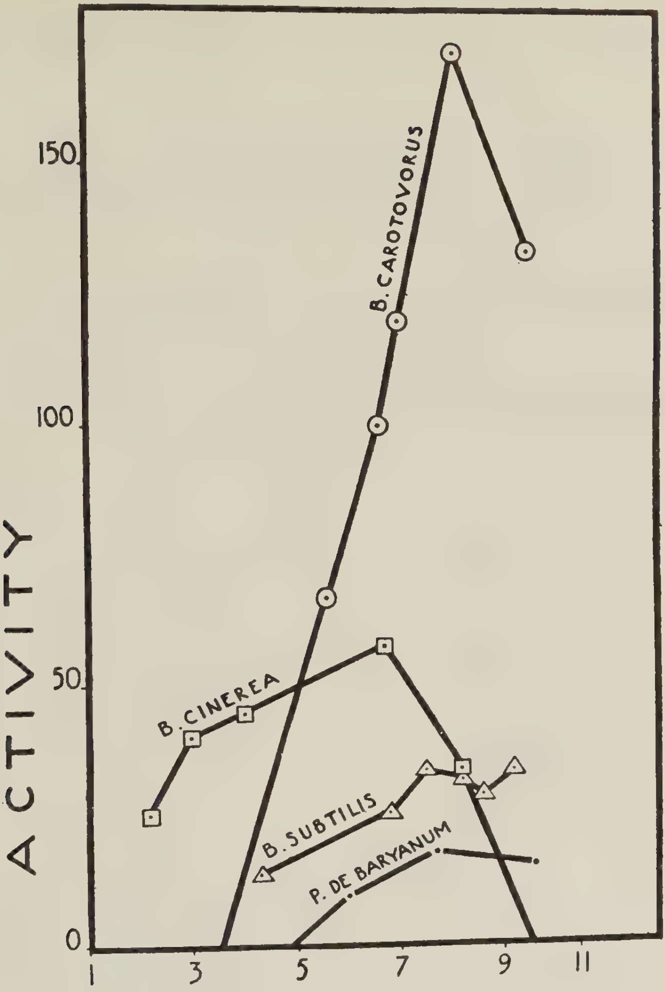

FIG. 4. Illustrating effect of pH on activity of enzyme of *B. carotovorus*, *B. subtilis*, *Botrytis cinerea*, and *Pythium de Baryanum*.

as to the exact trend of the curve for the Botrytis enzyme in acid solution, and while in fact the details may vary according to the strain of the fungus used or to the method of preparing the enzyme, all workers agree that the enzyme of Botrytis maintains a high degree of activity up to quite low pH values in the solution. Menon's results for Pythium agree closely with those obtained by the writer.

2. *Factors affecting enzyme production in cultural media.*

(*a*) *Effect of pH value and C/N ratio of synthetic cultural media.* The subject has been studied in fullest detail for *B. carotovorus*. For the sake of clearness the main conclusions will be stated first, and the illustrative detail given later.

Granted suitable conditions of aeration and temperature, the factor which is most important in determining whether a culture of *B. carotovorus* does or does not produce the enzyme in quantity is the pH value of the medium. Speaking generally, one may say that, whatever the initial pH of the medium, if the growth of the organism causes the pH value to move into the region pH 5·5–8·5 or thereby, good secretion of enzyme takes place. Active secretion is correlated with good growth of the organism, though from time to time enzymic solutions are obtained which show a greater activity than would correspond with the bacterial numbers present, and vice versa. The carbon/nitrogen ratio (C/N) of the initial medium is of considerable importance in connexion with growth and secretion of enzyme, but with slight qualification this factor appears to operate by determining the course of pH reaction.

In the experiments to be described, cultural media of various composition (more particularly with regard to the C/N ratio) were set up at different initial pH values, inoculated in a standard way with *B. carotovorus*, and from time to time examined as to

(*a*) drift of pH reaction,
(*b*) bacterial numbers present,
(*c*) enzymic activity of culture solution.

With regard to (*a*), the solutions were either unbuffered, with the result that the pH value would in some experiments move so rapidly to an extreme point that the multiplication of the organism was strongly checked; or they were buffered by the appropriate additions of phosphate mixtures, in order that the pH value should remain reasonably steady throughout the growth period. This was successfully accomplished in those experiments in which the C/N ratio of the medium was properly arranged.

The methods of determining (*b*) and (*c*) have already been described.

In the ultimate analysis one would presume that the enzymic activity of a culture solution depends on the three factors:

(1) Amount of bacterial growth.
(2) The rate of excretion per bacterium.
(3) The pH value of the solution at the time of testing.

The influence of the last factor can be determined by adjusting the solution tested to the pH value which was found in the preceding section to be optimal for enzymic activity; and such a method of standardizing was adopted. On the other hand, the separation of factors (1) and (2) was rarely possible. The method of testing enzymic activity which is employed is of little value when the solutions concerned are very weak, i.e. when the reaction time is much greater than twenty-four hours, and suitably active solutions are only obtained when there is vigorous bacterial growth. It is quite possible that the individual organism is actively secreting enzyme under conditions where the bacterial growth is severely checked, but the concentration of enzyme so produced may be too small to be determinable by the method available. It is only in the relatively rare cases where the curve of enzymic activity runs counter to the curve of bacterial numbers that one can suggest greater individual secretion in one set of conditions than in another.

After preliminary experiments the optimum pH value for the growth of *B. carotovorus* was determined by the type of experiment illustrated in Table I.

TABLE I

	After 1 day.		After 2 days.		After 4 days.	
Initial pH.	pH.	Bacterial nos. per c.c.	pH.	R.T. in hrs.	pH.	R.T. in hrs.
8·0	8·0	10×10^6	7·4	3	8·1	2·5
7·6	7·6	20×10^6	7·2	3	7·8	2·25
7·0	7·0	23×10^6	6·9	2·25	7·4	2
6·8	6·8	24×10^6	6·8	2·25	7·0	2
6·2	6·2	19×10^6	6·2	3·5	6·7	2

The medium used had the following composition: glucose 0·15 per cent., asparagin 0·5 per cent., $MgSO_4.7H_2O$ 0·075 per cent., KH_2PO_4 0·68 per cent. The different initial pH values were set up by the appropriate additions of N/5 NaOH.

Throughout the experiment the pH value did not change appreciably in each culture medium, i.e. the buffering was successful. After one day, at which time bacterial counts were made, no drift was apparent. The more alkaline media by and by became somewhat less alkaline, but by the fourth day had returned to approximately the initial pH values. This effect, which appears to be inevitable even in the best buffered solutions and which will be illustrated more strikingly in later tables, is probably due to the preferential attack by the organism of the glucose in the early stages. This would lead to formation of organic acids. As the glucose became depleted the organism would attack the organic acids and the carbon part of the asparagin, so that a drift to the alkaline side would set in. The same drift towards greater alkalinity, but without a preliminary drift to greater acidity, is shown in the media of lower initial pH (pH 6·2 and 6·8). This is what would be expected if the above explanation is a true one.

The optimum pH value for the growth of *B. carotovorus* is shown in Table I to lie within the range 6·2–7·6, and the best value suggested is pH 6·8.

In marked contrast to the results of Table I are those shown in Tables II and III. In both the latter the basal medium contained 0·075 per cent. $MgSO_4 . 7H_2O$ and 0·125 per cent. K_2HPO_4, i.e. the phosphate concentration was much less than that of the media in Table I. The buffering effect was thus much reduced. The concentrations of glucose and asparagin used were as follows:

	Glucose.	Asparagin.
Table II	0·1%	0·5%
Table III	0·2%	0·2%

TABLE II

	2 days.		4 days.		6 days.		10 days.	
Initial pH.	pH.	R.T. hrs.	pH.	R.T. hrs.	pH.	R.T. hrs.	pH.	R.T. hrs.
7·1	8·2	8	8·4	2·25	8·5	1·5	8·8	1·25
6·4	7·9	8	8·3	3	8·4	1·5	8·6	1·75
5·8	5·4	37	7·0	3·25	8·3	1·75	8·6	1·75
5·0	4·4	30	4·4	37	4·4	33	4·4	29

TABLE III

	4 days.		9 days.	
Initial pH.	pH.	R.T. in hrs.	pH.	R.T. in hrs.
8·3	7·9	9	8·5	1·75
7·6	6·4	10	8·4	2
7·1	5·3	>24	5·4	18
6·1	5·1	>24	5·2	>24
5·8	5·0	>24	5·2	>24
4·2	4·2	>24	4·2	>24

As a result of the low C/N ratio in the media of Table II a steady alkaline drift was shown in the culture solutions with initial pH 7·1 and 6·4. The solution with initial pH 5·8 first became more acid, then drifted to a pronounced alkaline point. On the other hand, the medium of initial pH 5·0 became too acid for active growth of the organism, an effect which was obvious from the solution remaining clear, whereas the others became distinctly turbid. High enzymic activity was shown in the first three but not in the last.

The relatively high C/N ratio of the media of Table III produced a tendency to development of acidity, so that it was only in the media of initial pH 8·3 and 7·6 that a final drift to an alkaline point occurred. All the remainder became acid and remained so till the end of the experiment. In all the media of Table III except the first two, bacterial growth was severely checked and enzymic activity was low.

Summarizing Tables I–III one can say that, independently of the initial pH value, good growth and enzyme production take place provided the medium is such that the reaction does not drift to an acid point and remain there. An acidity corresponding to about pH 5·4 represents the limit at which a reversal of drift can occur. At pH values less than this, growth is severely checked and little enzymic activity is developed; above this point the alkaline drift is able to set in, and when this is established there is a rapid development of enzymic activity (cf., in this connexion, the two days' and four days' old cultures of initial pH 5·8 in Table II).

The effect of the C/N ratio in a relatively unbuffered medium (K_2HPO_4 = 0·125 per cent.) is shown in detail in Table IV. All the media (*A*–*N*) had an initial pH of 7·0–7·3. In the table the upper row corresponding to each medium gives the pH drift, the lower row gives the reaction time of the enzyme.

TABLE IV

Media.	% glucose/ % asparagin.		2 days.	4 days.	6 days.	10 days.	16 days.
A	0/0·5	pH	8·3	8·6	8·7	8·7	8·9
		R.T.	15·5	3·5	3·25	2·5	2·25
B	0·02/0·5	pH	8·3	8·5	8·6	8·5	8 5
		R.T.	9·5	2·75	2·75	2	1·5
C	0·05/0·5	pH	8·3	8·4	8·5	8·5	8·7
		R.T.	9·5	2·5	2	2	1·5
D	0·02/0·2	pH	8·0	8·3	8·6	8 6	8·6
		R.T.	17	13·5	4·75	4·25	17
E	0·004/0·04	pH	8·3	8·3	8·2	8·2	8·2
		R.T.	33	20	18	21	22
F	0·1/0·5	pH	7·9	8·4	8·4	8·5	8·7
		R.T.	8·5	3	2	1·5	1
G	0·02/0·1	pH	7·9	8·4	8·4	8 4	8·4
		R.T.	18	17	16	7·75	20
H	0·004/0·02	pH	7·9	7·9	7·9	7·9	7·9
		R.T.	31	31	18	24	24
J	0·2/0·5	pH	5·3	5·5	5·6	8·2	8·4
		R.T.	45	37	19	2·25	1·25
K	0·02/0·05	pH	7·9	8·2	8·3	8·3	8·3
		R.T.	18	18	17	10	19
L	0·008/0·02	pH	7·9	7·9	7·9	7·9	7·9
		R.T.	33	30	18	24	26
M	0·5/0·5	pH	5·1	5·2	5·2	5·5	7·4
		R.T.	48	48	42	25	12
N	0·5/0·1	pH	4·8	4·8	4·8	4·8	5·5
		R.T.	48	48	48	48	46

The main features shown in Table IV are as follows:

(1) With media of the same high content of asparagin (*A*, *B*, *C*, *F*, *J*, and *M*), the pH drift is steadily to the alkaline side when the glucose concentration is low (*A*, *B*, *C*, and *F*). With higher glucose concentrations (*J* and *M*), the cultures first become acid, but later a reversal sets in, by the tenth day for *J* and by the sixteenth day for *M*. Good enzymic activity is shown in media *A*, *B*, *C*, and *F* from the fourth day onwards, whereas the same does not occur in *J* until the tenth day, and *M* is still weak even on the sixteenth day.

(2) With media of the same C/N ratio, but of different concentrations, e.g.

C, *D*, and *E* (ratio 1 : 10), the pH drift is the same in all, but enzymic activity progressively diminishes as total concentration decreases. The same holds for media *F*, *G*, and *H* (ratio 1 : 5). With *J*, *K*, and *L* (ratio 2 : 5), the activity of *J* is very low until the tenth day on account of a preliminary acid drift. When this drift is reversed, and conditions become alkaline, there is a relatively sudden increase in enzymic activity. With media *K* and *L* there is no such preliminary acid drift, whence it follows that, for the same ratio of carbon to nitrogen, increase of concentration accentuates the tendency to an acid drift.

(3) With media of the same glucose concentration (*B*, *D*, *G*, and *K*) the drift is much the same in all, but enzyme activity progressively diminishes with the fall in asparagin concentration.

A noteworthy feature of media with very low C/N ratio, e.g. *A*, is the deposition, after a few days, of crystals of ammonium magnesium phosphate.

Table V records the behaviour of media *A*, *F*, *J*, *M*, and *N* of Table IV, but with bacterial counts added for the sixth day.

TABLE V

		After 6 days		
Medium.	C/N ratio (%glucose/% asparagin).	pH.	bacterial no. per c.c.	R.T. hrs.
A	0/0·5	8·7	$2{\cdot}8\times10^{6}$	3·25
F	0·1/0·5	8·4	800×10^{6}	2
J	0·2/0·5	5·6	1700×10^{6}	19
M	0·5/0·5	5·2	$0{\cdot}003\times10^{6}$	42
N	0·5/0·1	4·8	$<10^{3}$	>48

The series of media *A*–*N* is one of increasing C/N ratio. Within this series there is a pronounced optimum for bacterial growth, viz. in medium *J*. Much greater enzymic activity, however, is shown in media *A* and *F*, which is the more remarkable in medium *A* when the relatively weak bacterial growth is borne in mind. Such a comparison is unfair in that the pH reaction of medium *J* is unfavourable to enzymic activity. When the comparison was made after adjustment of medium *J* to pH 8, which is optimal for enzyme activity, it was found that medium *A* was nearly twice as active as medium *J*. The obvious interpretation of this result is that much greater excretion of enzyme per individual bacterium took place under the conditions prevailing in *A* than in *J*.

The marked effect of the C/N ratio upon enzyme production illustrated in the foregoing, especially in Table IV, is not due in the main to carbon and nitrogen concentrations as such, but to the effect of the C/N ratio upon the pH value. If the media are sufficiently buffered to prevent extreme drifting of the pH value, very much the same enzymic concentrations are obtained for a wide range of C/N ratios in the culture medium. This point is illustrated in Table VI. It is only when the C/N ratio becomes too high, as in the last medium of the table, that the buffering has proved incompletely effective, with the result that a relatively weak enzymic solution is obtained.

Subsidiary experiments showed that, provided the pH value does not run to the acid side, a phosphate concentration of about 0·1–0·2 per cent. is optimal for enzyme production. The active secretion of enzyme which occurs in well buffered media, such as those of Table VI, is therefore not a direct effect of the high concentration of phosphate which is present.

TABLE VI

(*Solutions buffered with* 0·8 per cent. K_2HPO_4)

C/N ratio (% glucose/ % asparagin).	Initial pH.	After 3 days. pH.	After 3 days. R.T. in hrs.	After 6 days. pH.	After 6 days. R.T. in hrs.
0·02/0·5	7·2	8·4	1·16	8·6	<0·5
0·1/0·5	7·0	8·2	2	8·6	<0·5
0·25/0·25	7·1	7·8	1	8·0	<0·5
0·5/0·5	7·0	7·0	1·25	8·0	<0·5
0·5/0·1	7·1	6·2	3·75	5·8	1

The general conclusion from the experiments of Tables I–VI is that, apart from a stimulating effect of the nitrogenous constituent on enzymic production which may be noted in some cases, the controlling factor in enzymic production is the pH value arising in the medium. The C/N ratio of the culture solution is important in so far as it determines the drift of the reaction.

Table VII records an experiment in which *Botrytis cinerea* was grown on a basal medium consisting of 0·125 per cent. KH_2PO_4 and 0·075 per cent. $MgSO_4$, with different proportions of glucose and asparagin. A comparison of this Table with Table IV shows how differently the two organisms behave in respect of the pH drifts produced. On the two last media of Table VII, *B. carotovorus* would quickly develop a highly acid reaction which would inhibit growth and therefore produce very weak enzymic preparations.

TABLE VII

C/N ratio (% glucose/ % asparagin).	After 2 days Vol. of mycelium in c.c.	After 2 days Final pH.	After 2 days R.T. in hrs.
0/0·5	0·4	7·3	1·75
0·1/0·5	0·5	7·5	<0·5
0·2/0·5	0·6	7·7	<0·5
0·5/0·5	1·8	7·9	<0·5
1·0/0·5	6·2	7·7	<0·5

It is to be noted, however, that media of formula 0·1 per cent. glucose/0·5 per cent. asparagin and 0·2 per cent. glucose/0·5 per cent. asparagin give strong enzymic preparations with both organisms, so that the possibility of obtaining vigorous extracts from both on the same medium exists.

(*b*) *Effect of the nitrogenous constituent of synthetic media.* The availability of simple inorganic salts for enzyme production by *B. carotovorus* was also investigated. In salts, according as the nitrogen is present in the kation (e.g. NH_4Cl) or the anion (e.g. KNO_3), utilization of the nitrogen results in an accumulation of the anion or the kation with consequent development of an acid or an alkaline reaction. In view of the large extent to which the enzymic activity of *B. carotovorus* has been shown to depend upon the pH value, it was interesting to determine how far different inorganic sources of nitrogen had a direct effect on enzyme production, or alternatively an indirect one, through their influence on the pH drift.

Illustrative contrasting data are set out in Tables VIII and IX, in each of which the various nitrogenous compounds were used in equivalent proportions.

TABLE VIII

Basal medium: glucose, 0·2 per cent.; K_2HPO_4, 0·125 per cent.; $MgSO_4$, 0·075 per cent.

Conc. of N source: asparagin 0·2 per cent.; KNO_3, 0·307 per cent.; NH_4Cl, 0·162 per cent.

		After 4 days		After 9 days.	
Nitrogen source.	Initial pH.	pH.	R.T. hrs.	pH.	R.T. hrs.
Asparagin	7·1	5·3	>24	5·4	18
Potassium nitrate	6·9	6·4	14	5·5	12
Ammonium chloride	6·9	4·4	>24	4·2	>24

TABLE IX

Basal medium: glucose, 0·1 per cent.; K_2HPO_4, 0·8 per cent.; $MgSO_4$, 0·075 per cent.

Conc. of N source: asparagin 0·47 per cent.; KNO_3, 0·72 per cent.; NH_4Cl, 0·38 per cent.

		After 2 days.	After 3 days.	
Nitrogen source.	Initial pH.	pH.	pH.	R.T. hrs.
Asparagin	7·0	7·2	7·8	0·5
Potassium nitrate	7·3	7·2	7·7	0·75
Ammonium chloride	7·0	6·4	6·9	1

In the experiment of Table VIII, the high C/N ratio combined with the low degree of buffering resulted in an acid pH drift in all three media used, but this was greatest in the medium with ammonium chloride. All the media gave weak enzymic solutions, and this was especially so with the ammonium salt. On the other hand, the high degree of buffering and the low C/N ratio of the media of Table IX had the effect of maintaining a fairly steady pH value in all the cultures (though the acid drift was not suppressed in the medium with ammonium chloride), and good enzymic activity was shown in all. The effect of the nitrogenous constituent thus resolves itself, in the main, if not entirely, into a pH effect.

Parallel experiments with *B. subtilis* gave very similar results, except that the enzymic preparations obtainable from this organism are considerably weaker.

The effect of the nitrogenous compound on growth and enzyme secretion of *Botrytis cinerea* was studied by adding to a basal medium (glucose, 0·4 per cent.; KH_2PO_4, 0·125 per cent.; $MgSO_4$, 0·075 per cent.) equivalent concentrations of asparagin (0·047 per cent.), Witte's peptone, urea, potassium nitrate, sodium nitrate, and the following salts of ammonium: chloride, sulphate, phosphate (HPO_4), nitrate, and tartrate. The cultures were grown on horizontal glass plates, according to Brown's method, and at the end of two days enzymic activity, pH drift and amount of mycelium formed were determined. The main results may be summarized as follows:

1. Asparagin, peptone, and ammonium tartrate cause the pH value to drift to the region 6–7, and all produce very active enzyme solutions.

2. The ammonium salts of strong acids: chloride, sulphate, and nitrate, while giving growth comparable to that on asparagin, cause the pH value to move to the vicinity of pH 3 and enzymic activity is very low in all. With ammonium phosphate the effect is intermediate, both as regards pH and enzymic activity.

3. With urea and both nitrates growth was rather weak, and so also was enzyme production.

The data obtained indicated that when the pH value sank to the neighbourhood of 5·5 (as with ammonium phosphate) or lower (as with ammonium chloride), enzyme secretion was reduced. Thus the same rule appears to apply to *Botrytis cinerea* as to *B. carotovorus* (see p. 740). This result was rather unexpected for a fungus like *B. cinerea*, the optimum for growth of which is about pH 6.

(*c*) *Enzyme production on certain natural decoctions.* In the light of the preceding work with synthetic solutions of various compositions it was interesting to examine the course of the reaction produced by *B. carotovorus* and *B. subtilis* on natural plant extracts. These were prepared from potato, turnip, carrot, and apple in the usual way. Data illustrative of the results are included in Tables X and XI.

On potato extract a definitely alkaline reaction is produced which is quite favourable for the activity of the pectinase enzyme. Potato extract thus behaves like a synthetic medium with rather low C/N ratio. On this medium, *B. subtilis* reacts similarly, except that the enzymic preparations show less than one quarter the activity of those of *B. carotovorus* under the same conditions.

On the other hand, in the turnip extracts an acid reaction is developed. With *B. carotovorus* this reaches a pH value of about 5, which approaches the limit which this organism is able to tolerate. *B. subtilis* has a less pronounced acid-producing tendency, but here again the enzymic solutions obtained are weaker. Carrot extract shows the same features as turnip extract. In contrast to the preceding the apple extracts used were entirely unsuitable for the growth of *B. carotovorus*. In the first place the initial pH value was near 3·5, at which the organism would not grow; when the initial pH value was adjusted to neutrality, growth took place but the reaction rapidly moved to a low pH value (4·6), which stopped further growth. Even when the medium was buffered so that this extreme acid drift was

TABLE X

B. carotovorus on Potato Extract

	2 days.				7 days.			
		R.T. in hrs.				R.T. in hrs.		
Initial pH.	pH.	at prevailing pH.	at optimum pH.	*Growth.	pH.	at prevailing pH.	at optimum pH.	Growth.
5·2	5·3	40	15	+	8·4	<0·5	<0·5	++
5·6	6·6	2	1·5	++	8·4	<0·5	<0·5	++
6·9	6·4	1·5	0·75	++	8·4	<0·5	<0·5	++
8·1	8·0	0·5	0·5	++	8·5	<0·5	<0·5	++

* As determined by turbidity records; — indicates apparent absence of growth, + visible though poor growth, and + + profuse growth.

TABLE XI

B. carotovorus on Turnip Extract

	2 days.				7 days.			
		R.T. in hrs.				R.T. in hrs.		
Initial pH.	pH.	at prevailing pH.	at pH 7·0.	*Growth.	pH.	at prevailing pH.	at pH 7·0.	Growth.
4·3	4·3	>48	33	—	4·3	>24	>24	—
5·5	5·1	10	5	++	4·9	>24	6·25	++
6·1	5·2	6·5	3·25	++	5·0	11	3·5	++
7·3	5·2	10	3·5	++	5·2	5·25	1·25	++

* Notation as in Table X.

prevented, enzymic production was poor and was made appreciable only by the addition of an extra supply of nitrogenous substance. The composition of apple extract, and presumably, therefore, of apple tissue also, is of a kind that inhibits the growth and the parasitic mechanism of *B. carotovorus.*

SUMMARY

1. The optimum pH value for the pectinase enzyme of *Bacillus carotovorus* depends to some extent on the nature of the medium into which the enzyme is excreted. In young cultures the optimum pH of this enzyme is near to pH 8. If the culture medium is such that bacterial growth causes a pH drift to an acid point, or if the reaction is so maintained by appropriate buffering, the pH which is optimal for enzymic action likewise moves in the acid direction.

2. Subjection of the enzyme for some time to acid conditions displaces the optimum to a lower pH value.

3. The effects of the pH value on the activity of the enzymes of *B. carotovorus*, *B. subtilis*, *Botrytis cinerea*, and *Pythium de Baryanum* are compared and contrasted.

4. The production of active enzymic solutions by *B. carotovorus* is largely controlled by conditions which govern the rapid multiplication of the organism. On numerous media there is a tendency for an acid drift of the reaction, to be followed later by a reversed drift to alkalinity. Good growth and active enzyme solutions are obtained only when the pH drift towards acidity does not exceed pH 5·0.

5. On the basis of equal growth, cultures of *B. carotovorus* excrete pectinase enzymes more freely under alkaline than under acid conditions.

6. The chief effect of the carbon-nitrogen ratio of the cultural medium upon enzyme production is that it determines the direction and intensity of the pH drift. When this ratio is low, the reaction becomes alkaline and vigorous enzyme production occurs. With high C/N ratio, a high H-ion concentration which inhibits growth and enzyme production develops. Over a narrow range of media an inverse correlation between growth and enzyme production has been observed.

7. The effect of the nitrogenous constituent is also to a large extent an indirect one, though its influence on the reaction developed. With *Botrytis cinerea* nitrate appears to be a poor source of nitrogen from the point of view of enzyme production.

8. The behaviour of *B. carotovorus* and *B. subtilis* on vegetable decoctions has been studied and correlated with that on synthetic media.

The writer wishes to express his indebtedness to Professor W. Brown, at whose suggestion and under whose direction the work was carried out.

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The Breakdown of Cell Division in a Festuca-Lolium Derivative

BY

C. D. DARLINGTON

(*John Innes Horticultural Institution*)

AND

P. T. THOMAS

(*Department of Agricultural Botany, Aberystwyth*)

With Plate XXVIII and thirty Figures in the Text

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1. INTRODUCTION

IN recent years many sterile plants—hybrids, mutants, and others—have been studied in order to find out how and why their processes of pollen formation went astray. These studies have been particularly useful in showing the controlling agencies in cell division, more especially since they can be related to our detailed knowledge of chromosome mechanics. From such studies we can infer that an accurate co-ordination of these agencies is necessary to carry through the normal processes of cell division. It is the object of the present study to show how one of these, in some respects resembling previous cases and in others unique, can be used to define this co-ordination with greater exactness.

2. MATERIAL

Our material, for which we are indebted to Dr. T. J. Jenkin, is a single plant with 15 chromosomes derived by crossing *Lolium perenne* ($2n = 14$) by *Festuca arundinacea* ($2n = 6x = 42$), and backcrossing twice with the diploid parent as the male. It is therefore presumably a trisomic plant, closely related to *L. perenne*, which it resembles in external form. It produces, however, very little good pollen and the anthers fail to dehisce.

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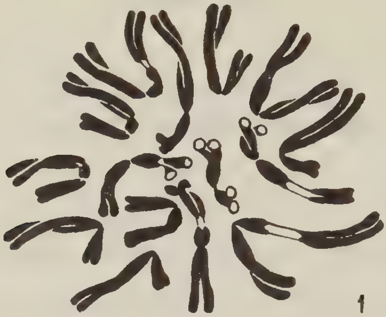
3. METHODS

The root-tips were fixed with 2 BD of La Cour (1937) and stained with gentian violet (Text-fig. 1).

The pollen mother-cells and pollen-grains were prepared by McClintock's acetic-alcohol and permanent acetocarmine method, but the material was transferred after one day from acetic alcohol to 70 per cent. alcohol in which it was left for two to three months. This special treatment was satisfactory in showing whatever structural differentiation of the cytoplasm was present at any stage of development.

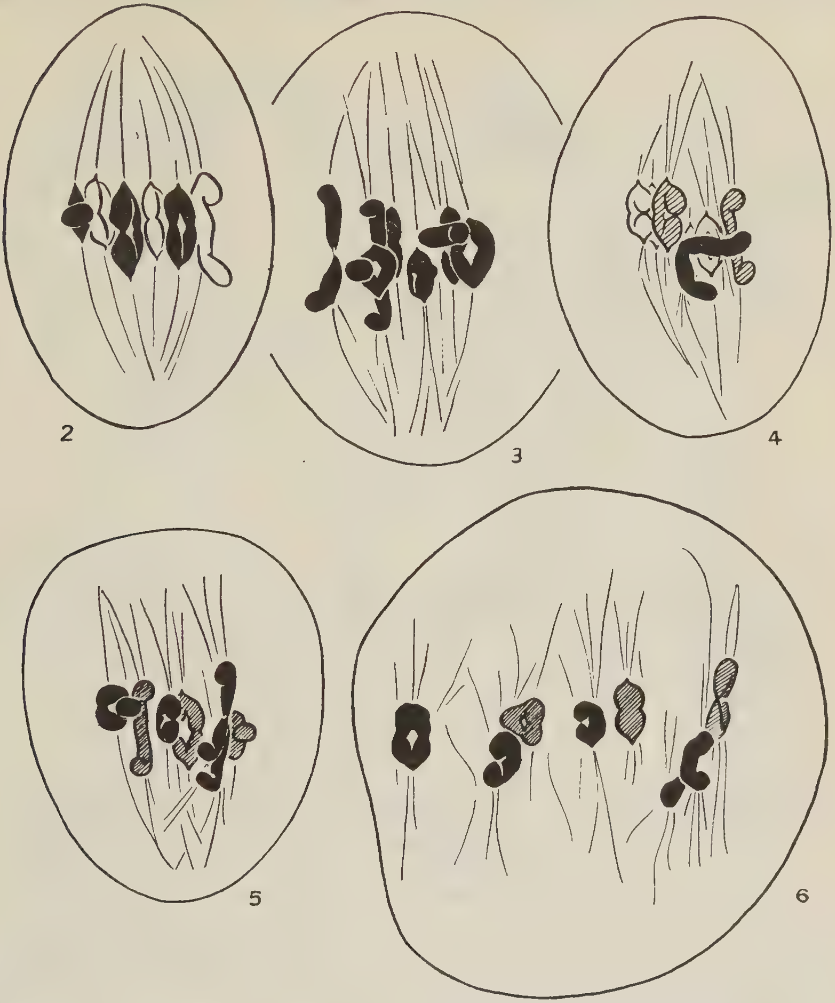


TEXT-FIG. 1. Mitosis in root-tip of the spindle-defective plant. $2n = 15$. Note: four chromosomes with nucleolar constrictions. × 3,000.

4. THE SPINDLE AND CHROMOSOME MOVEMENT

At the first metaphase the chromosomes are paired in the usual way by a number of chiasmata, one, two, or three, to give seven pairs and a univalent or six pairs and a trivalent. Once only we saw five pairs and three univalents. In the later stages, however, the cells show a transition from a type of spindle formation and chromosome movement that seems to be completely regular (Text-fig. 2) to an irregular type characteristic of this plant (Text-fig. 6 and Plate XXVIII).

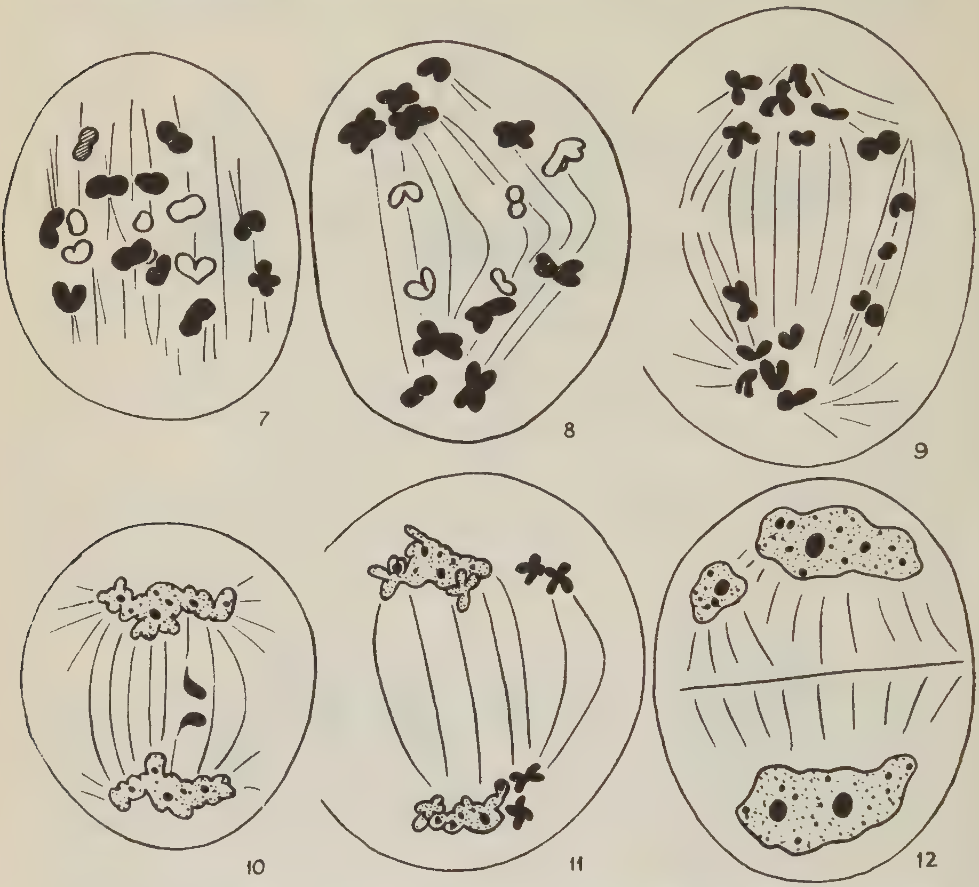
In the regular type it is impossible to say whether the spindle arises in the first instance from predetermined poles outside the nucleus or from the centromeres of the chromosomes inside the nucleus. But the result is that a compact spindle is formed at metaphase, with two approximately defined poles, and the bivalent chromosomes have their pairs of centromeres co-orientated on the arcs of this spindle. Irregularity shows itself in its mildest form either by a lack of compactness at one or both poles (Text-figs. 3 and 5) or by lack of orientation of one or more bivalents. Where the lack of compactness is found it is noticed that the striations of the spindle diverge from the centromeres, some of them running criss-cross instead of converging on a pole. The extreme form is represented in Text-fig. 6 by a somewhat flattened cell. This flattening in itself is not without significance, because the lack of compactness of the spindle probably makes the chromosomes more easily separable in the act of smearing. However that may be, we notice that



Text-figs. 2–6. Side views of first metaphase in the pollen mother-cell showing a gradation from a compact to a discontinuous or incompact spindle. The extra chromosome is shown as a univalent in Fig. 3 and a trivalent in Figs. 4, 5, and 6; it is not shown in Fig. 2. × 1,500.

although the chromosomes seem to have formed separate spindles, these separate spindles have arranged themselves more or less parallel with the chromosomes in an equatorial plate. The chromosomes are more contracted than in normal cells.

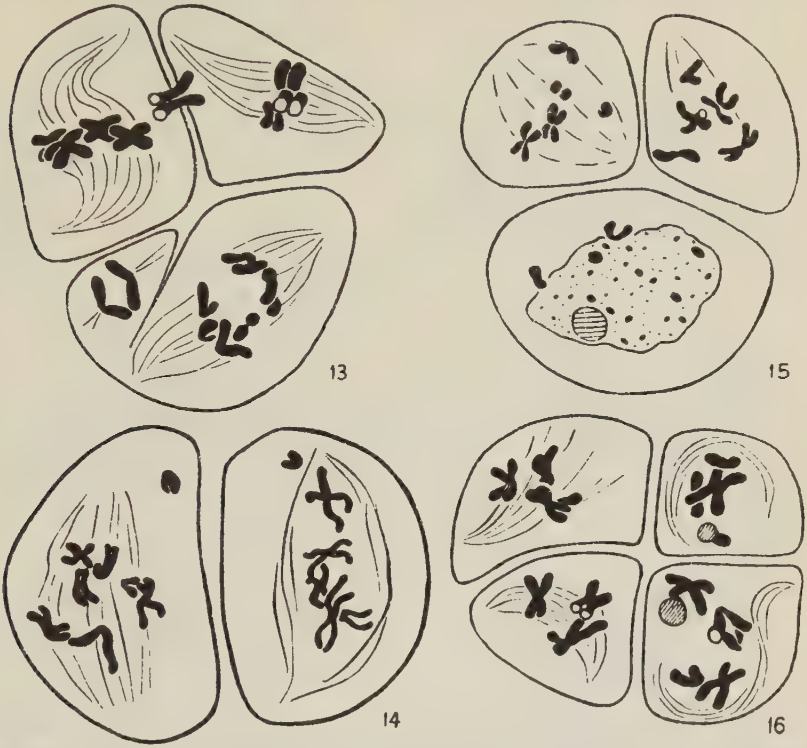
The most characteristic result of the *incompact* spindle is the divergence of one of the two daughter groups of chromosomes to form two daughter nuclei, so that three nuclei in all are formed at telophase. In the course of this formation the spindle may be bent or very much contorted. The spindle may also diverge on both sides, in which case four nuclei are formed at the



Text-figs. 7–12. First anaphase to telophase showing the effect of discontinuity in the pindle. Note lagging univalent in Fig. 10 and the formation of an extra nucleus in Figs. 11 nd 12. × 1,500.

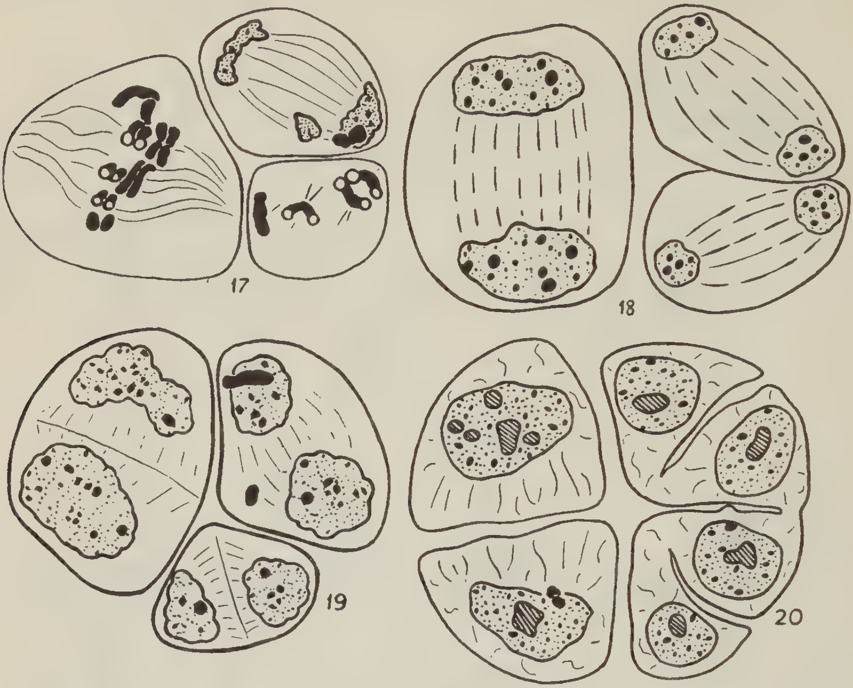
nd of the first division (Text-figs. 13, 16). Between the three or four nuclei hat are formed, cell-walls begin to develop. The one that cuts across the riginal spindle is always perfect, but those that separate the two parts of one aughter group are often imperfect. (Text-figs. 13 and 20).

We therefore find ourselves at a stage corresponding to the dyad or interhase stage with two, three, or four nuclei in two, three, or four cells, according to the degree of abnormality of the first division. Now whatever this egree, we find that the second division within each daughter cell may be ither regular or irregular, and may be of an entirely opposite character in the products of the same pollen mother-cell, e.g. Text-figs. 14, 15, and 16. Thus we may have a regular spindle with regular separation of either a single chromosome or any number up to six or seven (Text-fig. 13, right-hand cells, or Plate XXVIII, Fig. 4, showing abnormal orientation in the two cells) or, on the other hand, the spindle may show a certain incompactness, as in the first division (Text-fig. 14, left hand) or it may be bent (Text-fig. 13, left hand). Again, it may be unipolar (Text-fig. 16) or, finally, it may pass completely around the chromosomes as though avoiding them (Text-figs. 14 and 16). In these last anomalous cells the nucleoli often persist from interphase. Such cells are found always in the same anther with normal products of division which are at a later stage of development (cf. Plate XXVIII, Fig. 5). We even find nuclei still in an arrested interphase when their sisters in the same mother-cell are passing through a regular second metaphase (Text-fig. 15).

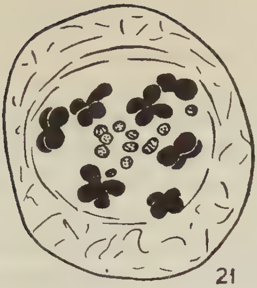


TEXT-FIGS. 13–16. Second metaphase. Fig. 13. Four nuclei have been formed at first telophase. The two lower ones are in anaphase, imperfect cell-wall between. One upper one has a bent spindle. Fig. 14. Two nuclei have been formed. One shows a semi-normal spindle, the other an unattached spindle. The univalent has lagged and divided at the first division. Fig. 15. Lower nucleus has remained in interphase. Fig. 16. One unipolar spindle, one bent spindle, and two unattached spindles with nucleoli persisting. × 1,500.

While the incompact types of abnormal spindle perhaps give rise to regular telophases or restitution nuclei, it seems that the *unattached* spindles, which avoid the chromosomes, give rise to a peculiar kind of degeneration. The spindle gradually disappears, but the chromosomes, instead of reverting to normal nuclei, have become even more condensed; two chromatids of each remain held together at their centromeres as in a late interphase between the first and second divisions. A nuclear boundary is formed around the chromosomes and small nucleoli appear (Text-figs. 21, 22, 24) which condense into irregular masses attached to the chromosomes (Text-figs. 23, 25, 26). The appearance of nucleoli at a moment when condensation increases seems to be contrary to the supposition of those who infer a genetic continuity between nucleoli and chromatin. The abnormal nuclei seem to resemble the so-called 'diakinesis-like' nuclei found by Kihara and Lilienfeld (1936) in an Aegilops-Haynaldia hybrid, where the earlier details of cell division are not clear. In our plant, however, as we shall see, there is reason to believe that the resulting pollen grains do not develop a thick wall as they do in the Aegilops hybrid (cf. also Beadle (1932*a*) on precocious pollen grains in va_2 plant of *Zea Mays*).



Text-figs. 17–20. Second anaphase and telophase in cells which have produced three daughter nuclei. Fig. 17. Smallest cell with two chromosomes having separate spindles. Fig. 18. Normal second telophase with six nuclei. Fig. 19. Cell-wall formation beginning. Later stage. Fig. 20. Still later stage, but cell-wall formation imperfect. Figs. 17–19, × 1,500: Fig. 20, × 1,700.



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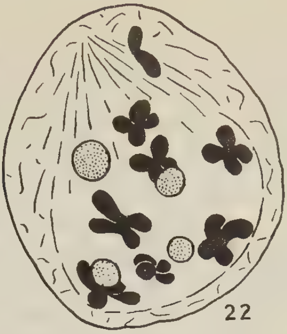

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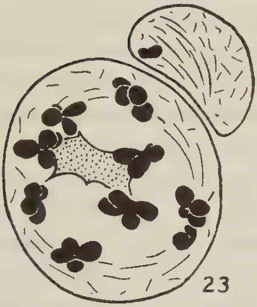

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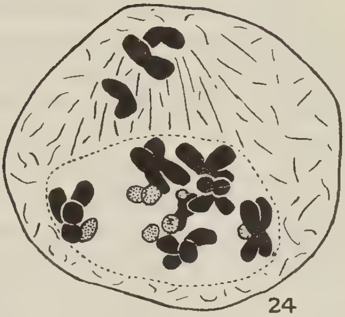

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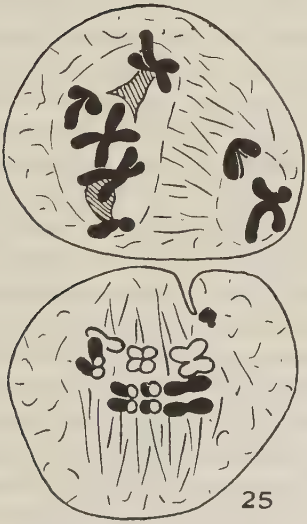

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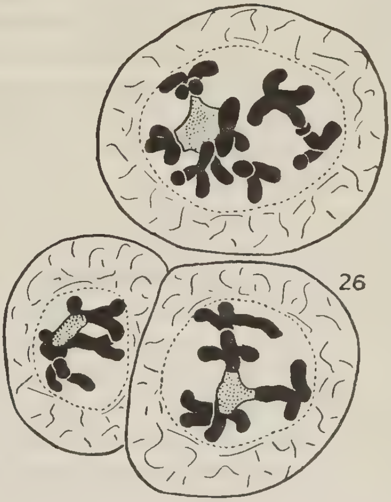

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TEXT-FIGS. 21–6. Cells from an abortive second division, where an unattached spindle has been followed by a relapse into an interphase-like stage, showing the gradual disappearance of the spindle, formation and coalescence of somewhat irregular nucleoli and development of boundary between nucleus and cytoplasm. Figs. 21–4. Detached cells. Fig. 25. The two daughter-cells, lower one semi-normal. Fig. 26. The common type with three cells, with 7½, 4½, and 3 chromosomes. × 1,700.

5. The Origin and Behaviour of the Spindle

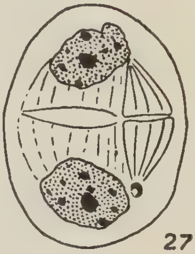
The spindle-defective plant, like many other trisomics, is without doubt genotypically abnormal in a way that affects the conduct of cell division. Since the pairing of its chromosomes is regular, its abnormality must be due essentially to the particular genotypic combination it has received from its mixed ancestry, and not to any direct effect of hybridity. The error arises not, as in a structural hybrid, from a defect in chromosome homology, but as in polymitotic and asynaptic maize, through the action of an exceptional genotype. As in all other such cases the irregularity produced occurs in various degrees. The abnormal genotype is less protected against environmental variations than the common or normal genotypes.

In order to understand how the spindle defect arises we must consider how a normal spindle develops. In many organisms a visible centrosome controls the formation of the spindle. In the higher plants no such visible agent is present outside the nucleus. An invisible external agent may, however, be inferred from the formation of cell-walls setting apart portions of the cell devoid of chromosomes in the pollen mother-cell divisions of certain grass hybrids and haploids (e.g. Kagawa and Chizaki, 1934; Chizaki, 1934). This inference depends on the assumption of a control of cell-wall formation by the spindle which we shall consider later. The defective spindles in our plant, however, provide direct evidence of the *external* as well as *internal* agents determining the spindle, since the defective spindle arises in two ways corresponding to defects in these two agents: viz. as an incompact spindle from the separate action of the centromeres and as a contorted or unattached spindle from the separate action of something outside the chromosomes and the nucleus. Either method is inadequate in giving a regular separation of the products of division.

If there are two separate agents of cell division, how can we explain correlated errors in both of them in the same plant and even in the same cells? Clearly their mutual adjustment must be at fault. We have noticed that the abnormal cells are delayed as compared with their regular neighbours. Such a delay is found in many other cases of abnormal cell division (cf. Darlington, 1937, Table LXII). It is therefore most likely that the fault lies in the timing adjustment of the two agents. Thus we may suppose that when the external agents are too late, the chromosomes themselves establish the spindle independently, with the incompact result we have seen. After this frustration of the external agents at the first division, they act too soon in the second; the chromosomes are therefore too late and the spindle develops externally, avoiding the chromosomes, which cannot take their proper part in its development. This conclusion is strengthened by two particular observations. First, the chromosomes in the compact spindle are super-contracted. They have been delayed in developing the spindle on their own, with the same result as in Lathyrus, where the delay is general (Fabergé, 1937; Upcott, 1937).

Secondly, the spindle stretches in the second-division cells where the chromosomes are not ready for it, just as it does in the first meiotic divisions where there has been a failure of pairing and where, as we know, the chromosomes, being univalent, are delayed in orientation on the spindle.

Variation in the proper time co-ordination of the two agents of cell division is shown at meiosis in the grasshopper Trimerotropis (Carothers, 1936) where bivalents, having their centromeres already co-ordinated with the spindle (as in Text-fig. 2), lie side by side with a bivalent with its own spindle (as in Text-fig. 6). Such slight deviations from a normal adjustment are probably very common, and a split anaphase may also be found exceptionally in otherwise regular meiosis (e.g. in Campanula, Darlington and Gairdner, 1937; cf. also Beadle, 1932*a*, Fig. 1). They are inherent in any failure of pairing at meiosis. Lagging chromosomes necessarily modify the anaphase spindle in which they lie (Text-fig. 27, cf. Vignoli on Agave, 1936). A timing irregularity (such as the precocity of the external agents of division which determines meiosis) can take effect only where there are two potentially independent processes which have to be co-ordinated in time for an effective division. The identification of the agents responsible for these processes thus establishes the sequence of events in the defective plant, and hence the necessity of co-ordination in the normal plant. We may say that a regular development of the spindle depends on the co-operation of external and internal agents, pole-determinants, and centromeres, co-ordinated in time as well as in space.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TEXT-FIG. 27. Anaphase in an unrelated Lolium hybrid, showing a double spindle. ×800.

The failure of co-ordination in the defective plant, as in so many other cases of abnormal meiosis, does not affect the regularity of mitosis. The reason is clear. The restriction of the defect to meiosis is not due to the special genetic consequences of meiosis, but to its special physiological causation. The particular time co-ordination of centromere and poles is different in mitosis and meiosis. The system which is adequate for one breaks down when it has to deal with the other.

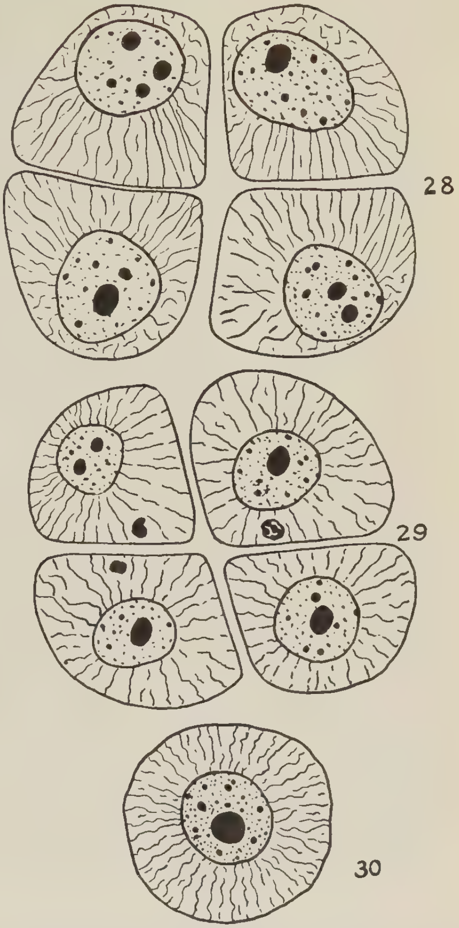
When we consider in detail how the present defect works, we see that the extra-nuclear agents responsible for forming the spindle are not of the character of the centrosomes found in animals and lower plants. The method of division is, for example, essentially different from that with tripolar spindles where three centrosomes are present as in the tetraploid fern ally Psilotum (Okabe, 1929) or in certain Hemiptera (Bowen, 1922). Thus one daughter group of chromosomes gives rise to one or two nuclei and one or two cells according to the degree of compactness of the spindle, and if two nuclei are formed at one end of the spindle, a new spindle may be developed in each of them at the second division as effectively or almost as effectively as if there had been only one. The spindle-forming agents outside the nucleus must therefore be supposed to exist as diffused particles which coalesce or congregate at the moment when spindle poles are normally formed and exist separately at other times. Hence we find a multipolar stage at early metaphase in some plants. The pole-determinants have the function of centrosomes and possibly their continuity, but they do not preserve this continuity as single and visible coherent bodies.

The behaviour of the incompact spindle is interesting from another point of view. Its only serious error is in the divergence of the separating groups of chromosomes. A parallel co-ordination of the separate centromere spindles arises inherently without the co-operation of the external agent. Such a co-ordination of separate spindles is normal in certain Hemiptera (e.g. Schrader, 1931). We thus see how certain Protozoa can dispense altogether with the activity of the centrosomes in spindle formation. If the cell itself were sufficiently compact it would be possible for our incompact centromere spindle to give rise regularly to two daughter nuclei. The assistance of centrosomes or of undefined organizers becomes indispensable in cells having more than a certain volume in proportion to their spindles. So it is, we may suppose, that in Artemia, while the centrosomes co-operate in ordinary spindle formation in the open cell, an intranuclear spindle can nevertheless act efficiently without them during the maturation of the egg (Gross, 1935; cf. Darlington, 1937). Thus we find in different organisms and in different stages of their development various ways of co-ordinating poles and centromeres, each adapted to the special conditions of cell-size, available materials, and special requirements of growth and differentiation.

6. The Spindle and Cell-wall Formation

It has for some time been clear that the spindle, or a spindle-like differentiation of the cytoplasm, has two functions, the first in relation to chromosome movement and the second, in plants, in relation to cell-wall formation. The same spindle may serve one of these functions or both in succession. In the first pollen mother-cell division, particularly in dicotyledons, in the endosperm of angiosperms (Jungers, 1931) and in the embryos of certain gymnosperms, the original spindle fails to give rise to a cell-wall, but a spindle-like striation develops independently of nuclear division and some time after it, to form cell-walls between nuclei. In many cases, as in the separation of the generative cell in the pollen (Piech, 1928) or the simultaneous formation of walls between all four nuclei in the pollen mother-cell, a spindle associated originally with chromosome movements extends or is supplemented to form a cell-wall or cell-boundary over a much wider region than that covered by the original spindle. The same principle applies to the trinuclear cells formed by our compact spindles, where a cell-wall develops between nuclei that have not been separated at any time by a mitotic spindle. The origin and structure of these secondary spindles are then concealed under the learned name 'phragmoplast'.

The apparent lack of relationship of the mitotic spindle and cell-wall formation in such instances has led Kihara and Lilienfeld (1936) to dispute the existence of any necessary connexion between them. Such a view can be upheld no longer. It is true that the relation of a spindle structure to the delimitation of cells in animals is far from clear, although in the fungi a connexion has been established in certain cases (e.g. by Harper, 1896, and Fraser and Brooks, 1909). But in the higher plants the direct observations of Belar (1929) and others on the movements of the spindle during and in relation to the formation of the wall have left no doubt of the spindle's secondary function. Moreover there is a correlation between the effects of the chromosomes on the anaphase spindle and the formation of cell-walls giving supernumerary cells around lagging chromosomes at the first anaphase of meiosis (Darlington, 1936). The present observations go farther. They provide, in the first place, a different kind of evidence of the same relationship, which seems to be equally conclusive. Defects of the spindle and of chromosome movement are associated with defects in the formation of the cell-wall, as Moffett found in Kniphofia (1932). But it is particularly from a study of the internal structure of the cytoplasm during the formation of both normal and defective cell-walls that we see the close relationship of the spindle with them.



Text-figs. 28–30. Normal tetrads and young pollen grains, where cell-wall formation is active, showing the development of spindle-like differentiation in the cytoplasm, accompanied by movement of the nucleus to the centre and uniform cell-wall formation. × 1,700.

In the young tetrad (Text-fig. 28) we see strongly marked striations, the relics of the second-division spindle, connecting the daughter nuclei with the newly-formed cell-wall. Later, the nuclei move towards the middle of the cell and the striations spread around them radially in all directions (Text-fig. 29). Finally the young and separated pollen grain which is actively thickening its cell-wall shows a maximum and uniform striation of its cytoplasm, the whole of which may now be described as a phragmoplast or secondary spindle (Text-fig. 30).

When, on the other hand, we turn to relapsed nuclei with condensed chromosomes, we find no signs of orientated striation in the cytoplasm (Text-fig. 26, Plate XXVIII). These young pollen grains will presumably die without any further development of a cell-wall.

It therefore seems that an orientation of the cytoplasm is in plants generally necessary for the regular and orientated deposition of particles to make the cell-wall. Further it seems that this orientation of the cytoplasm is conveniently co-ordinated with that necessary for the separation of the chromosomes in nuclear division in most cells. Orientation may also arise either for the original formation or for the later thickening of the cell-wall, without any direct relation with a mitotic spindle or with the movement of the chromosomes. In a cell with a resting nucleus, such an orientation of the cytoplasm will be radial. One pole must be the surface of the nucleus, the other the surface of the cell, just as in the unipolar spindles produced experimentally in various animals or naturally in Sciara, where one pole is the centrosome and the other the cell-wall.

It may seem surprising that we should assume that the poles may take so many forms, concentrated on centrosomes or centromeres, diffused on nuclear or cell-walls. During mitosis, however, we have detailed evidence of the substitution of one kind of pole for another in the transition from metaphase to anaphase when the centrosome or polar spindle becomes transformed into a centromere or cylindrical spindle. The separate action of the individual centromeres or chromosomes has already been shown from the effect of lagging chromosomes in determining the formation of the separate cell-walls around small supernumerary 'microcytes' (Darlington, 1936). We have also found evidence of this where lagging chromosomes rejoin the main group at telophase. In the otherwise normal plant referred to earlier, two separate elements may be seen in the telophase spindle where lagging chromosomes have clearly developed their part of the spindle after the main body, and the one has consequently been superimposed on the other (Text-fig. 27). Such lagging is probably the result of non-congression of one or more bivalents at first metaphase, as may happen wherever the bivalents are crowded on the plate.

In fact there seems no reason to doubt that a spindle structure may be determined by several kinds of pole and may serve the function of regulating movement whether for growth or cell division. But, whatever its origin or function, its structure is essentially the same as that of the mitotic spindle and must therefore be understood in terms of our special knowledge of the mechanics of the mitotic spindle. We must regard it as due to an orientation of the contained water determined by a state of stress between electrically charged surfaces.

The great diversity of results obtained by the spindle's action will necessarily depend on the size and dispersion of the charges, the distances separating them, the shape of the molecules determining the orientation of the associated water, and the kinds of smaller molecules that are free to move in the channels created by their orientation. In short, we must look upon the spindle as the result of a special method the living substance has of arranging itself, a method which comes into play under various conditions and with various effects. Since we can understand these conditions and effects best as they take place during cell division, it is by studying the laws regulating cell division that we are likely to find the key to the most general problems of cell behaviour.

SUMMARY

1. A trisomic pollen-sterile Lolium-Festuca hybrid derivative ($2n = 15$) has spindle formation and cell division in the pollen mother-cells varying from a regular to an anomalous and irregular type.
2. At first metaphase the centromeres of the chromosomes develop separate spindles and daughter groups diverge at anaphase to give three or four nuclei instead of two.
3. At the second division, spindles are formed, which either stretch or bend before the chromosomes form a plate, or actually move round the nucleus, never coming into relationship with the chromosomes, which then become more condensed and relapse into a telophase-like condition.
4. Thus it follows that there must be external agents necessary for normal spindle formation and that these act too late for the chromosomes at the first division, too early for them at the second. The balance necessary for the regular accomplishment of cell division is upset. Without the co-operation of the external organizers the spindle is discontinuous and *incompact*; without the co-operation of the chromosomes the spindle is *unattached*; it lengthens and bends and finally disappears without effect on the chromosomes.
5. The external and internal agents of cell division are therefore, as assumed by the precocity theory of meiosis, independent in causation although related in effect.
6. Since three or four spindles can arise from one incompact one, the external organizers, although perhaps permanent, must be dispersed and cannot be coherent bodies. In this important respect they differ from centrosomes.
7. Further, the formation and development of the cell-wall are associated, in the resting stage as well as during mitosis, with a differentiation of the cytoplasm corresponding in its characteristic artefacts to that which produces the mitotic spindle. The two poles are then the nuclear surface and the cell-wall.
8. A spindle structure, therefore, may be determined by several kinds of poles, and may have two important regulative functions, but its structure is essentially the same and must therefore depend on the physical conditions already identified in dividing cells.

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EXPLANATION OF PLATE XXVIII

Illustrating Dr. Darlington and Dr. Thomas's paper on 'The Breakdown of Cell Division in a Festuca-Lolium Derivative'

All microphotographs are of pollen mother-cells and (except Fig. 2), are from the defective plant.

Fig. 1. Separate centromere spindles at first metaphase (cf. Text-fig. 6). × 800.

Fig. 2. Normal metaphase spindle in a different *Lolium* hybrid with delayed congression of one bivalent, for comparison with Fig. 1. × 850.

Fig. 3. Second divisions in abnormal cells (middle, cf. Text-fig. 17). × 800.

Fig. 4. Normal second divisions showing merely a twisted relative orientation of spindles. × 800.

Fig. 5. Normal tetrads side by side with two abnormal triads of first telophase (cf. Text-fig. 12). × 500.

Fig. 6. Triad, partly in interphase and partly in second metaphase (cf. Text-fig. 15). × 1,000.

Fig. 7. Regressive interphase and second metaphase (cf. Text-fig. 25). × 1,000.

Fig. 8. Regressive interphase and unipolar microcyte: third cell lost (cf. Text-fig. 23). × 1,200.

Fig. 9. Regressive separated tetrad cell with no orientation of cytoplasm (cf. Text-fig. 26). × 1,200.

Fig. 10. Active young pollen-grain (cf. Text-fig. 30). × 900

Fig. 11. Group of tetrads, normal with orientation clear, slightly abnormal with orientation less clear (cf. Text-figs. 19 and 29). × 800.

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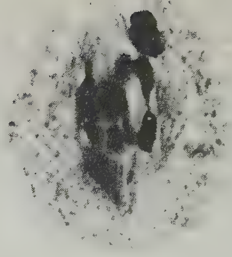

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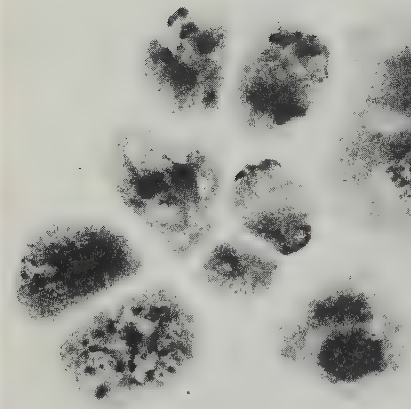

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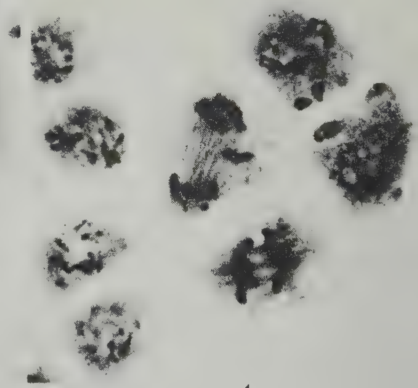

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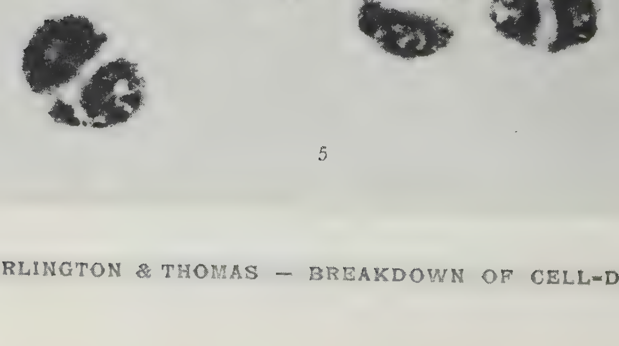

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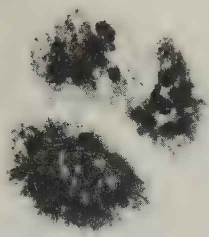
DARLINGTON & THOMAS — BREAKDOWN OF CELL-DIVISION.



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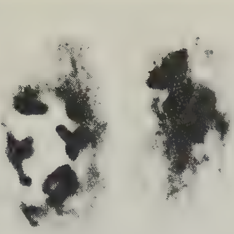

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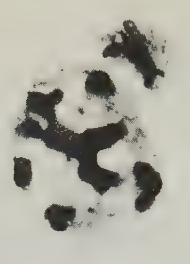

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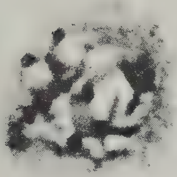

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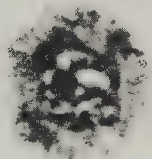

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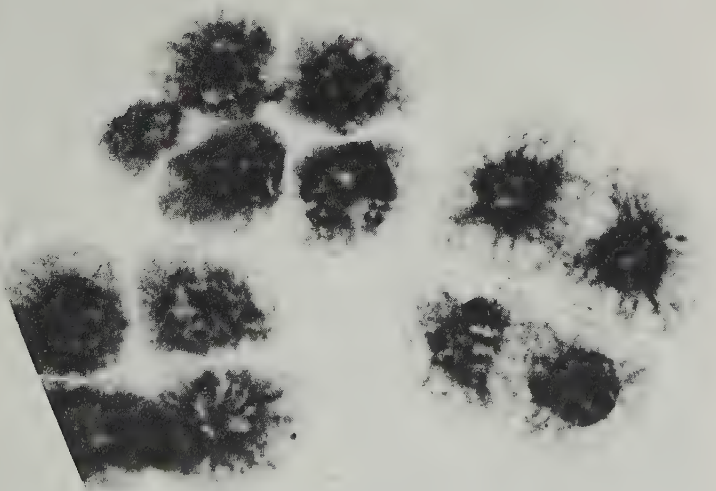

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Huth, Stubbs X., Kent.

A Study of the Epidemiology and Control of Helminthosporium Disease of Oats

BY

A. E. MUSKETT

(*Department of Agricultural Botany, Queen's University, Belfast*)

With Plates XXIX and XXX and eight Figures in the Text

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INTRODUCTION

THE disease of oats caused by the fungus *Helminthosporium Avenae* Eid., and known as Seedling Blight, Leaf Stripe, or Leaf Spot, according to the symptoms produced, may in some seasons cause severe damage to the crop. It appears to be exclusively seed-borne and infected seed, when sown, may give rise to seedlings affected with the primary phase of the disease. This phase normally exhibits the symptoms of Seedling Blight or Leaf Stripe, and it is here that severe crop damage may occur. Blighted seedlings are killed by the disease either before they emerge or shortly after their emergence from the soil, while those which show severe striping of the first or first and second leaves either succumb during their early stages of development or grow on to produce plants of impaired vigour. Crop attack by the primary phase of the disease is one of the causes which may result in a thin stand of seedlings. The secondary phase of the disease, which generally takes the form of Leaf Spot and ultimately leads to the infection of the seed, arises through the infection of the leaves by conidia produced by the fungus on seedlings attacked by the primary phase. The onset of the secondary phase may cause no appreciable crop injury but is of importance in that by its agency infected seed is produced.

The primary phase of the disease may be prevented by the disinfection of infected seed prior to sowing (MacKay, 1933; Muskett and Cairns, 1932; O'Brien and Prentice, 1930), and it is therefore desirable that any seed disinfectant recommended for use with oats should procure a satisfactory control of Helminthosporium disease in addition to that of other seed-borne diseases. For some years past an attempt has been made in Northern Ireland

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to estimate the relative values of various seed disinfectants in preventing this disease by carrying out, annually, a series of field trials. In spite of the fact that heavily infected samples of seed were used for this work the results obtained were often disappointing owing to the low incidence of the disease occurring in the control plots. In some years an accurate assessment of the values of various disinfectants could not be made and it was found necessary to extend the trial period over several seasons before reliable results could be obtained.

It therefore became apparent that if a method could be evolved whereby the evaluation of seed disinfectants could be arrived at with greater speed and accuracy than that obtained through the accepted medium of field trials, a considerable advance would have been made. The success of such a method, in so far as it involves the evaluation of the disinfectant by making a count of the diseased seedlings produced by infected seed, must rely upon the ability to produce a high and, if possible, maximum incidence of the disease at will, which in turn depends upon a knowledge of the epidemiology of the disease. An epidemiological study was undertaken during the two years 1934–5, and the following is an account of the work done and the results which were obtained.

EXPERIMENTAL TECHNIQUE

The problem was approached by growing seedlings under varying conditions of soil moisture and soil temperature, which were controlled as closely as possible until the plants had developed the maximum incidence of primary infection. Round tins, 22 cm. diam. × 11·5 cm. high, such as are employed for the baking of cakes, were used as pots, ordinary flower-pots being unsuitable on account of their porous nature and the consequent difficulty of maintaining a constant soil moisture during the experimental period. This size of tin was chosen as it was found to provide suitable conditions for the raising of 100 seedlings to the desired stage of growth. The plants were grown on an open veranda erected along the wall of one side of the flat roof of the Agricultural Building. The roof of the veranda was sloped at such an angle so as to allow a maximum of light to reach the plants, and projected just far enough to shelter them from rain. These growth conditions were chosen as those most nearly approaching the natural which would fulfil the experimental requirements.

The seed. The variety Potato was selected for the work as it is widely grown and susceptible to the disease. Through the courtesy of Messrs. McGill and Smith, Ayr, Scotland, two samples of Pure Line Potato oats were obtained, one of which came from a stock of heavily infected seed and the other from a lightly infected stock. Both samples were used and will be referred to as Potato A and Potato B respectively. The seed was stored under dry conditions in the laboratory, sample A being used in both years.

When disinfection of the seed was carried out prior to sowing, this was done by shaking the seed with the requisite quantity of an organic mercury dust of proved worth in a plugged conical flask for a period of five minutes.

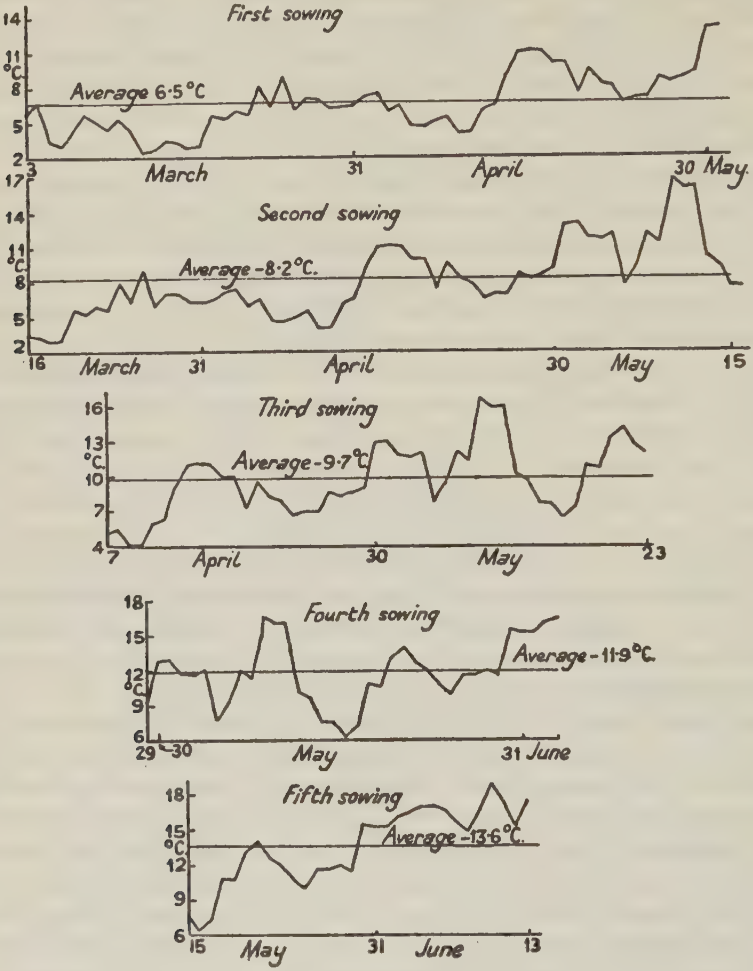
The soil. In 1934 two soils of very different types were used. One, a heavy rich loam, was obtained from the plant pathological field station at the Agricultural Research Institute, Hillsborough, Co. Down, and the other, a light sandy loam, from the lands of the Plant Breeding Division at Stormont. These will be referred to subsequently as the Hillsborough and Stormont soils. As soil type did not appear to influence appreciably the results obtained in 1934, Hillsborough soil only was used in 1935.

Temperature control. Self-recording thermometers were kept among the pots in the veranda during the whole period of the investigation, so that a complete record of the temperature variation was obtained. The problem of raising the plants under varying temperature conditions was met by making sowings at different times of the year. The first sowing was made in early March and was followed by subsequent sowings at intervals of approximately fourteen days until the middle of May. This ten-week period gave not only substantial variations of temperature conditions but also represented the time of year during which the sowing of spring oats would be carried out in farm practice. The temperature registered was that of the air surrounding the pots, and this may be taken as approximating very closely to that of the soil, for it was found by repeated tests that the temperature of the soil in the pots at the depth of sowing never varied from that of the surrounding air by more than 1° C. The mean temperature for each particular sowing was calculated by averaging the temperatures recorded at intervals of two hours during the whole of the experimental period, this being equivalent to the taking of twelve temperature readings for each period of twenty-four hours.

Temperature curves for each of the ten sowings made during the two years are given in Text-figs. 1 and 2. The mean daily temperatures recorded here were arrived at by averaging the twelve two-hourly readings taken over each twenty-four-hour period. It will be seen that in 1934 a very considerable range of temperature variation occurred between the first and fifth sowings; the mean for the first sowing was 6·6° C. and that for the fifth sowing 13·6° C., the means for the other sowings occupying intermediate positions. In 1935 the range was more limited, the mean temperature for the first sowing being 8·5° C. and that for the fifth 11·3° C. The mean for the fourth sowing was actually slightly higher than that for the fifth—it being 11·4° C.

Within the total experimental period occupied by each sowing there was a wide temperature variation due to the alternation of warm and cool periods and warm days following cold nights. These wide variations occurring in any one sowing raised the question as to whether the mean temperature calculated over the complete experimental period presented a true picture of the variation of disease incidence with temperature. Apparently the assessment is sufficiently accurate for the purpose under consideration, as when temperature means calculated over periods of ten and twenty days immediately following sowing were substituted for those calculated for the whole experimental period the order of the results was not appreciably affected.

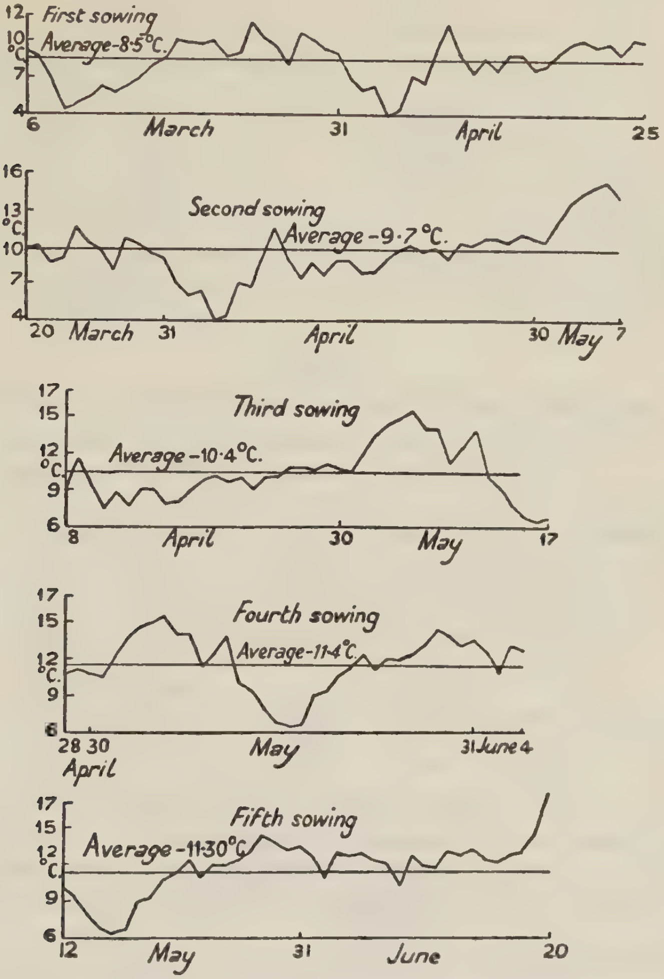
The duration of the experimental period for each sowing varied with the temperature conditions. Whereas a period of sixty days was necessary to bring the seedlings to the desired stage of growth under low temperature conditions, it was reduced to thirty days in the case of later sowings.



TEXT-FIG. 1. Temperature curves for 1934 sowings.

The mean temperatures obtaining for the various sowings and the duration periods of the experiments are given in Table I.

Moisture control. Three degrees of soil moisture were used, the object being to study the incidence of the disease under wet, moist, and dry soil conditions. After bringing in the soil from the field it was air dried by spreading it in a thin layer on the floor of a heated greenhouse, after which it was sieved, mixed thoroughly, and stored in bags until required. When needed for use a sufficient quantity was spread in a thin layer on a hard floor and watered with a hose, thoroughly turning the soil all the time, until the desired moisture content had been reached. Dry soil conditions corresponded to those likely to obtain in a season of drought, moist soil conditions to those of an ideal growing season, and wet soil conditions to those resulting from a wet season in land which is not well drained. Samples were taken immediately after preparation for moisture estimation and portions of them were retained for purposes of comparison when fresh batches of soil were being prepared. After a little experience it was found quite practicable to judge the moisture content



Text-fig. 2. Temperature curves for 1935 sowings.

sufficiently accurately for the work by feeling the texture of the soil with the hand.

Soil-moisture estimations were made according to a modification of the box method described by Hall (1921), the results of the determinations being set out in Table II.

TABLE I

1934.				1935.			
No. of sowing.	Sowing date.	Mean temp. ° C.	Duration of experiment (days).	No. of sowing.	Sowing date.	Mean temp. ° C.	Duration of experiment (days).
1	Mar. 3	6·6	60	1	Mar. 6	8·5	51
2	Mar. 16	8·2	61	2	Mar. 20	9·7	49
3	Apr. 17	9·7	47	3	Apr. 8	10·4	40
4	Apr. 29	11·9	36	4	Apr. 28	11·4	38
5	May 15	13·6	30	5	May 12	11·3	40

TABLE II

Estimations of Soil Moisture. Results expressed as Percentage Saturation of Soil

		1st sowing.	2nd sowing.	3rd sowing.	4th sowing.	5th sowing.
				1934		
Hillsborough soil:	wet	72·2	69·1	68·1	69·1	65·4
,, ,,	moist	45·8	46·0	42·8	41·1	39·0
,, ,,	dry	26·8	25·9	28·4	26·8	26·3
Stormont soil:	wet	75·2	65·1	73·4	62·3	65·4
,, ,,	moist	53·0	47·4	44·9	35·5	41·5
,, ,,	dry	27·2	25·1	24·7	26·6	26·2
				1935		
Hillsborough soil:	wet	72·3	68·2	74·3	70·3	76·1
,, ,,	moist	52·5	47·7	55·0	50·7	55·9
,, ,,	dry	27·0	26·7	28·0	33·6	29·5

It will be seen from these results that the judging of the soil moisture content by feeling its texture proved to be practicable and that the variations occurring in the results for any one grade of moisture content are insignificant when compared with the major differences occurring between the grades.

Method of sowing. (See Pl. XXIX.)

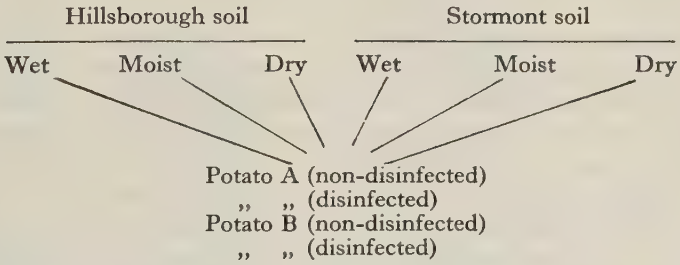
When preparing the pots for sowing, the same quantity of soil was weighed into each, the quantity varying for the different soils used and for the different moisture contents. A decimal scale (sensitive to 1·0 gm.) was found to be very suitable for this purpose. Each pot was filled to within about 1·5 in. (4·0 cm.) from the top, and sufficient of the soil was put aside for covering the seed after sowing. The soil in each pot was lightly compressed with a wooden dumper and the weight checked again before sowing.

After preparation the pots were covered to prevent loss of moisture from the soil and sowing was carried out on the following day. The even distribution of the 100 seeds sown in each pot was secured by sowing through holes, cut equidistantly, in a disc of three-ply wood made so as to fit neatly inside the pot. Before sowing, holes of equal depth were made to receive the seed by the use of small wooden punches designed to fit the holes in the wooden disc. In order to avoid the rubbing of dust disinfectant from the seed, forceps were used for the sowing operation. The seed was sown at a depth of approximately 1·25 cm. (0·5 in.). After sowing, the seeds were carefully covered with soil of the same moisture content as that contained in the pot, it being assured by weighing that the same amount of covering soil was used for each pot.

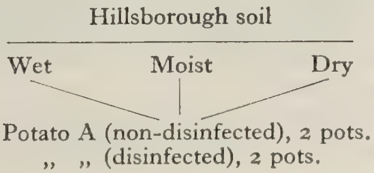
Care after sowing. Immediately after sowing, the pots were placed in position in the veranda and the moisture content of each was kept constant by watering on alternate days. When watering, each pot was brought up to the weight it registered immediately after sowing, the decimal scale employed in filling the pots being used for this purpose.

EXPERIMENTAL LAY-OUT

In 1934 the unit selected for trial under each set of conditions was one pot containing 100 plants. As the work planned for the year involved the preparation of 120 pots it was not possible to employ larger units. Two soils were used (Hillsborough and Stormont), two seed samples (Potato A and Potato B), and parallel sets of non-disinfected and disinfected seed were set up. Each of the five sowings comprised 24 pots, and the experimental lay-out for each of the sowings may be diagrammatically represented as shown below:



In 1935 only one soil type (Hillsborough) and one seed sample (Potato A) were used, the experimental unit for this year comprising two pots each containing 100 plants. The experimental lay-out of each of the five sowings for this year may be diagrammatically represented as follows:


Hillsborough soil
Wet
Moist
Dry
Potato A (non-disinfected), 2 pots.
,, ,, (disinfected), 2 pots.

RESULTS

Preliminary tests indicated that the maximum incidence of the primary phase of the disease had occurred by the time the growth in length of the second leaf of the seedling equalled that of the first, and each experiment was concluded when this stage had been reached. Results were recorded by cutting each seedling, examining it for the presence of the disease, and placing it in one of two categories—healthy or diseased. The diseased seedlings were further separated into two groups—those likely to survive and those likely to die as the result of attack. Diseased seedlings were often recognized by their non-erect habit of growth, while the crops produced by non-disinfected seed were characterized in general by uneven growth and disorderly appearance, when compared with those grown from disinfected seed where control of the disease had resulted. These symptoms were particularly marked during the earlier stages of growth. During the course of the work all stages of attack were observed and the findings of O'Brien and Prentice (1930), O'Brien and Dennis (1932), and Turner (1933) confirmed. Gross distortion of the diseased seedlings was most pronounced under dry soil conditions. The unequal growth resultant upon the attack being more pronounced or confined to one side of the plant caused the seedling to bend sharply over soon after emergence, and in many cases such seedlings grew parallel with and pressed to the soil surface. Plants attacked in this way were killed either before or soon after the appearance of the first leaf. The difficulty experienced in definitely diagnosing suspicious markings on the coleoptile as due to Helminthosporium attack resulted in plants so marked being classed as healthy unless, of course, the markings were accompanied by striping of the leaf.

The results are set out in Tables III–VII and are expressed diagrammatically in Text-figs. 3–8. Photographs of the diseased populations occurring in the different sowings of Potato oats A in Stormont soil in 1934 are shown in Pl. XXX. The information given in the tables includes figures for total seedling emergence, healthy seedlings, the pre-emergence phase of the disease, the post-emergence phases, total disease, and the estimated mortality among seedlings showing post-emergence attack, all expressed as percentages of the total number of seeds sown. The extent of the pre-emergence phase of the disease was taken as the difference between seedling emergence occurring in crops from non-disinfected seed and disinfected seed. As it was not possible to diagnose every case of non-emergence in the non-disinfected seed as being due to Helminthosporium attack the exact accuracy of this estimation is not claimed, but it will be seen from the tables and diagrams that in most cases the inclusion of the pre-emergence estimation with the post-emergence phases gives a more consistent series of results than when the post-emergence phases are considered by themselves.

Summary of results. The incidence and intensity of Helminthosporium disease of oats decrease under wet and moist soil conditions with a rise in the

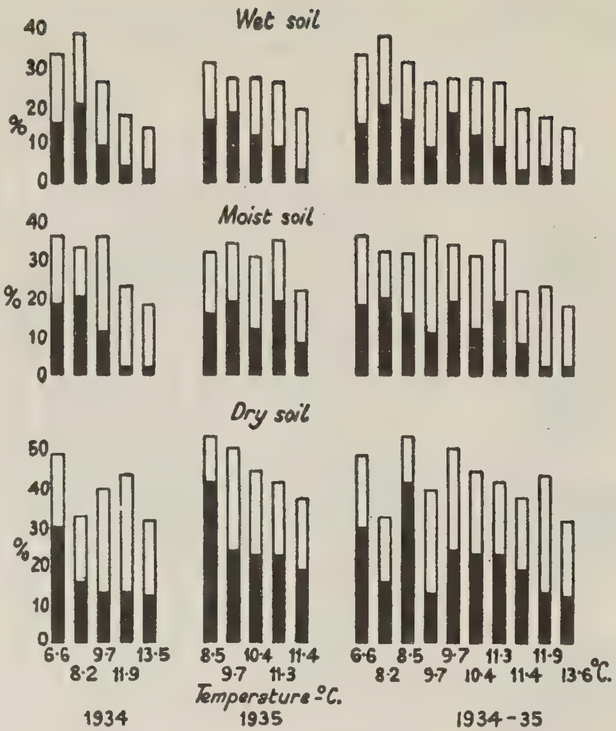


FIG. 3.

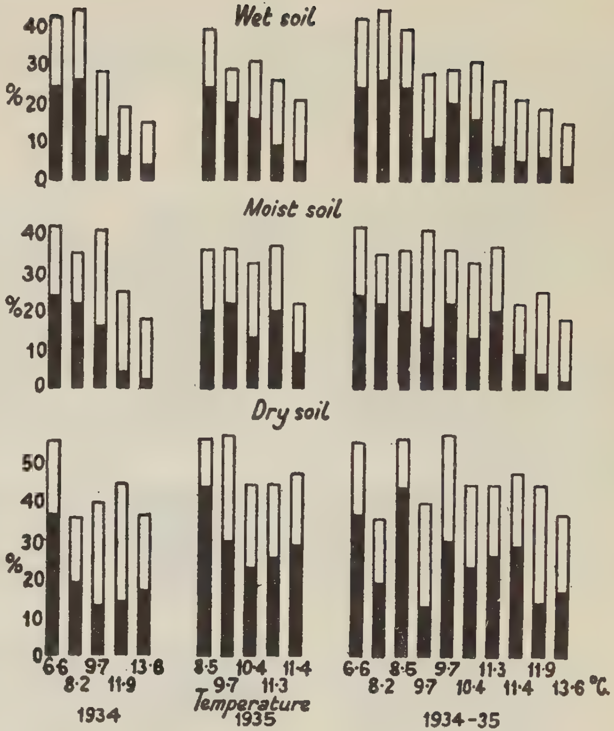
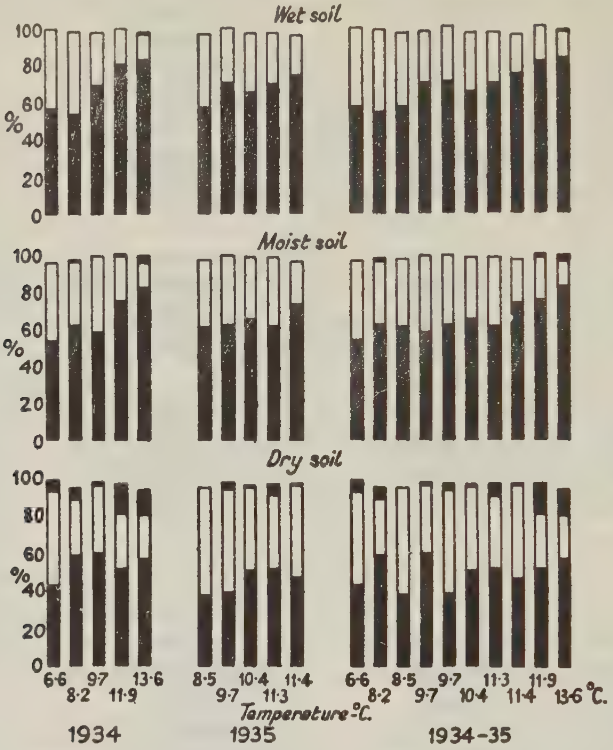


FIG. 4.

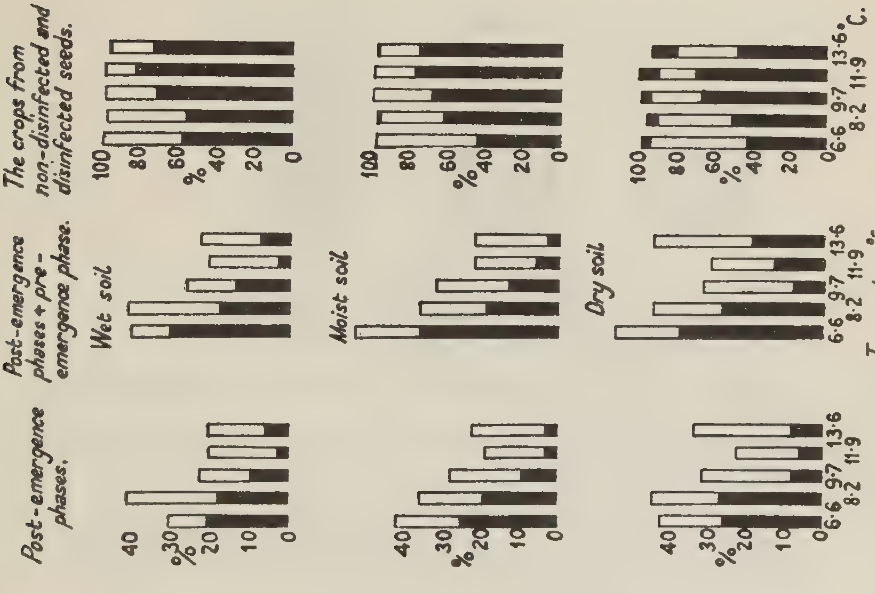
TEXT-FIG. 3. Potato oats A, Hillsborough soil, 1934–5: Helminthosporium disease, Post-emergence phases. The total length of each column represents the percentage of post-emergence phases of Helminthosporium disease. The blackened portion represents the estimated percentage of seedlings likely to succumb to the attack. The five columns to the left of each series show the results obtained in 1934; the five centre columns for 1935, while the ten columns to the right show the results for both years considered together.

TEXT-FIG. 4. Potato oats A, Hillsborough soil, 1934–5: Helminthosporium disease, Post-emergence phases+Pre-emergence phase. The total length of each column represents the percentage of post-emergence phases to which has been added the percentage of the pre-emergence phase estimated as the difference in emergence between seedling stands from non-disinfected and disinfected seeds. The blackened portion represents the total estimated mortality, which includes the pre-emergence phase in addition to those likely to succumb through post-emergence attack. The five columns to the left of each series show the results obtained in 1934; the five centre columns those for 1935; while the ten columns on the right show the results for both years considered together.

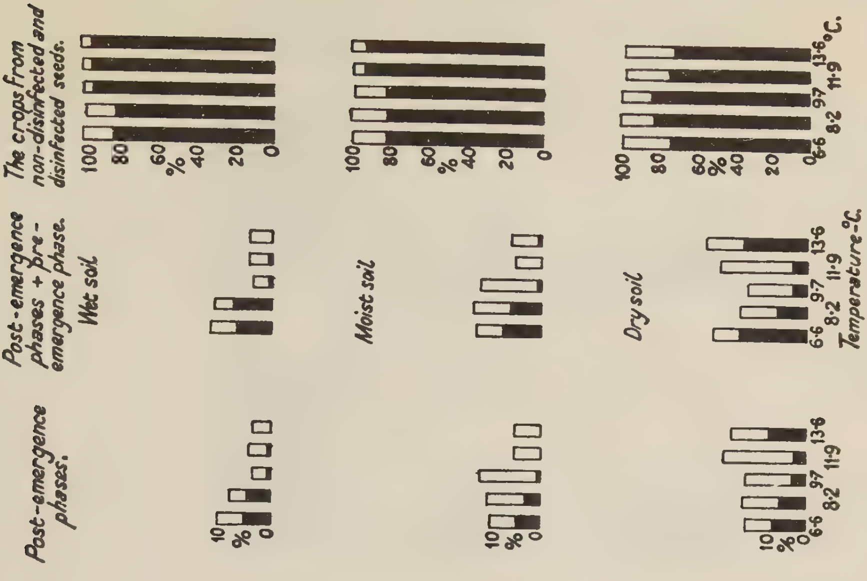
mean temperature ranging from 6·6 to 13·6° C. The disease is aggravated by dry soil conditions, where the same decrease with rise in temperature is not observed due to the poor growth conditions furnished by the dry soil. In general, as the conditions favouring the rapid and healthy growth of the oat plant are improved so is the incidence of the disease decreased. Using a sample of oats (Potato A) containing approximately 60 per cent. of infected seeds it has been possible, to quote extreme cases, to produce 56 per cent. of diseased seedlings under cold dry soil conditions, whereas only 15 per cent. of the seedlings were diseased when the soil conditions were wet and warm.



TEXT-FIG. 5. Potato oats A, Hillsborough soil, 1934–5: Helminthosporium disease, The crops from non-disinfected and disinfected seeds. The height of each column represents the percentage of seedling emergence resulting from the sowing of disinfected seeds. The blackened lower portion represents the percentage of healthy seedlings produced by non-disinfected seeds. The upper blackened portion of a column represents the percentage of the disease occurring in seedlings raised from disinfected seeds when disinfection did not secure complete control. It will be noticed that this is almost completely confined to the dry soil series, reference to which is made in the text. The five columns to the left of each series show the results obtained in 1934; the five centre columns those for 1935, while the ten columns to the right show the results for both years considered together.

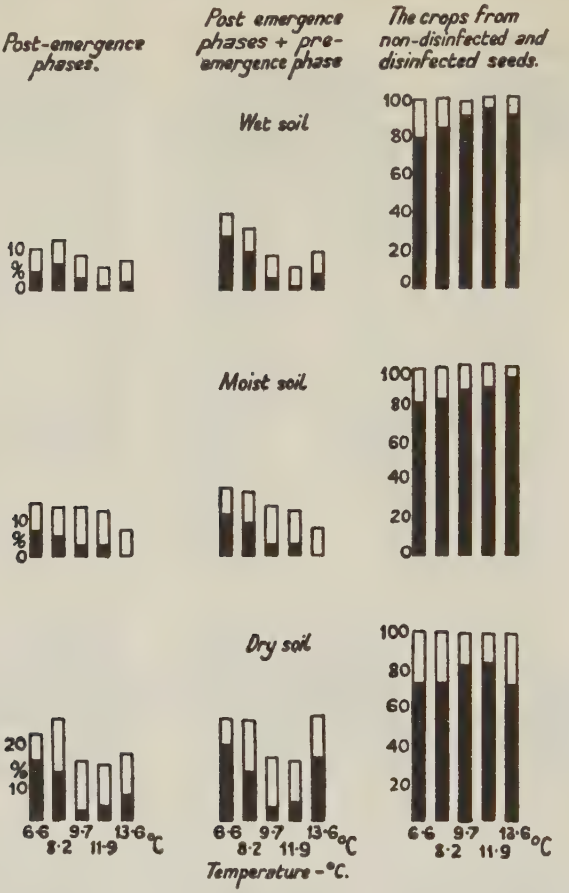


TEXT-FIG. 6. Potato oats A, Stormont soil, 1934: Helminthosporium disease. The explanation provided for Text-figs. 3, 4, and 5 also apply to Text-fig. 6.



TEXT-FIG. 7. Potato oats B, Hillsborough soil, 1934: Helminthosporium disease. The explanation provided for Text-figs. 3, 4, and 5 also apply to Text-fig. 7.

The pre-emergence phase of the disease is most marked under cold wet conditions of growth. It also occurs at high temperatures when the soil is dry, but the accuracy of the results obtained in such cases is vitiated by a tendency to irregular germination.



TEXT-FIG. 8. Potato oats B, Stormont soil, 1934: Helminthosporium disease. The explanation provided for Text-figs. 3, 4, and 5 also apply to Text-fig. 8.

The results obtained hold for seed samples of Potato oats whether heavily or lightly affected with the disease.

Variation in soil type does not influence the incidence of the disease to nearly the same extent as variation in the temperature and moisture content. Its effect probably varies only in so far as its heating properties and moisture-retaining propensities are concerned.

Seed disinfection with an organic mercury dust of proved worth secured almost perfect control of the disease under all conditions except in the case of dry soil subject to relatively high temperature.

TABLE III

Potato A, Hillsborough Soil, 1934: Helminthosporium Disease

The results are expressed as percentages of the number of seeds sown.

Sowing.	Seed treatment.	Wet soil.						Moist soil.						Dry soil.					
		Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence mortality (estimated).	Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence mortality (estimated).	Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence mortality (estimated).
First (6·6° C.)	Non-disinfected	89	56	9	33	42	15	89	53	6	36	42	18	92	43	7	49	56	30
	Disinfected	98	98	0	0	0	0	95	95	0	0	0	0	99	93	0	6	6	0
Second (8·2° C.)	Non-disinfected	91	53	6	38	44	20	94	61	2	33	35	20	92	59	3	33	36	16
	Disinfected	97	97	0	0	0	0	96	95	0	1	1	0	95	89	0	6	6	0
Third (9·7° C.)	Non-disinfected	94	68	2	26	28	9	93	57	5	36	41	11	100	60	0	40	40	13
	Disinfected	96	96	0	0	0	0	98	98	0	0	0	0	98	96	0	2	2	0
Fourth (11·9° C.)	Non-disinfected	96	79	2	17	19	4	97	74	2	23	25	2	96	52	1	44	45	13
	Disinfected	98	98	0	0	0	0	99	98	0	1	1	0	97	81	0	16	16	0
Fifth (13·6° C.)	Non-disinfected	95	81	1	14	15	3	99	81	0	18	18	2	89	57	5	32	37	12
	Disinfected	96	95	0	1	1	0	98	94	0	4	4	0	94	80	0	14	14	0

TABLE IV

Potato A, Hillsborough Soil, 1935: Helminthosporium Disease

The results are expressed as percentages of the number of seeds sown.

Sowing.	Seed treatment.	Wet soil.						Moist soil.						Dry soil.					
		Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence mortality (estimated).	Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence mortality (estimated).	Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence. mortality (estimated).
First (8·5° C.)	Non-disinfected	87	56	8	31	39	16	92	60	4	32	36	16	93	39	2	54	56	42
	Disinfected	95	95	0	0	0	0	96	96	0	0	0	0	95	95	0	0	0	0
Second (9·7° C.)	Non-disinfected	96	69	2	27	29	18	95	61	3	34	37	19	91	39	6	52	58	24
	Disinfected	98	98	0	0	0	0	98	98	0	0	0	0	97	94	0	3	3	0
Third (10·4° C.)	Non-disinfected	91	64	4	27	31	12	96	64	1	32	33	12	96	51	0	45	45	23
	Disinfected	95	95	0	0	0	0	97	97	0	0	0	0	96	95	0	1	1	0
Fifth (11·3° C.)	Non-disinfected	95	69	0	26	26	9	96	60	1	36	37	19	94	52	3	42	45	23
	Disinfected	95	95	0	0	0	0	97	97	0	0	0	0	97	91	0	6	6	0
Fourth (11·4° C.)	Non-disinfected	93	73	1	20	21	4	95	73	0	22	22	9	86	48	10	38	48	19
	Disinfected	94	94	0	0	0	0	95	95	0	0	0	0	96	96	0	0	0	0

TABLE V

Potato A, Stormont Soil, 1934: Helminthosporium Disease

The results are expressed as percentages of the number of seeds sown.

Sowing.	Seed treatment.	Wet soil.						Moist soil.						Dry soil.					
		Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence mortality (estimated).	Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence mortality (estimated).	Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence mortality (estimated).
First (6·6° C.)	Non-disinfected	88	57	10	31	41	21	85	43	11	42	53	25	85	42	12	43	55	26
	Disinfected	98	98	0	0	0	0	96	96	0	0	0	0	97	93	0	4	4	0
Second (8.2° C.)	Non-disinfected	97	55	0	42	42	18	97	61	0	36	36	19	95	50	0	45	45	27
	Disinfected	96	96	0	0	0	0	96	94	0	2	2	0	95	89	0	6	6	0
Third (9·7° C.)	Non-disinfected	93	70	4	23	27	10	95	67	4	28	32	9	98	66	0	32	32	8
	Disinfected	97	97	0	0	0	0	99	99	0	0	0	0	98	93	0	5	5	0
Fourth (11·9° C.)	Non-disinfected	100	81	0	21	21	3	95	76	3	19	22	3	92	69	7	23	30	6
	Disinfected	98	97	0	1	1	0	98	98	0	0	0	0	99	89	0	10	10	0
Fifth (13·6° C.)	Non-disinfected	93	72	2	21	23	6	96	74	0	22	22	3	81	47	11	34	45	8
	Disinfected	95	94	0	1	1	0	96	95	0	1	1	0	92	79	0	13	13	0

TABLE VI

Potato B, Hillsborough Soil, 1934: Helminthosporium Disease

The results are expressed as percentages of the number of seeds sown.

Sowing.	Seed treatment.	Wet soil.						Moist soil.						Dry soil.					
		Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence mortality (estimated).	Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence mortality (estimated).	Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence mortality (estimated).
First (6.6° C.)	Non-disinfected	97	83	2	14	16	7	95	82	4	13	17	6	89	73	9	16	25	9
	Disinfected	99	99	0	0	0	0	99	99	0	0	0	0	98	98	0	0	0	0
Second (8·2° C.)	Non-disinfected	93	82	4	11	15	6	96	81	4	14	18	4	98	82	1	17	18	7
	Disinfected	97	97	0	0	0	0	100	100	0	0	0	0	100	100	0	0	0	0
Third (9·7° C.)	Non-disinfected	99	94	0	5	5	1	98	82	0	16	16	1	99	83	0	16	16	4
	Disinfected	99	99	0	0	0	0	98	98	0	0	0	0	99	99	0	0	0	0
Fourth (11·9° C.)	Non-disinfected	100	94	0	6	6	1	100	93	0	7	7	0	96	74	1	22	23	3
	Disinfected	99	99	0	0	0	0	99	99	0	0	0	0	97	97	0	0	0	0
Fifth (13·6° C.)	Non-disinfected	99	94	1	5	6	0	99	92	1	7	8	0	91	71	7	20	27	10
	Disinfected	100	100	0	0	0	0	100	100	0	0	0	0	98	98	0	0	0	0

TABLE VII

Potato B, Stormont Soil, 1934: Helminthosporium Disease

The results are expressed as percentages of the number of seeds sown.

Sowing.	Seed treatment.	Wet soil.						Moist soil.						Dry soil.					
		Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence mortality (estimated).	Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence mortality (estimated).	Seedling emergence.	Healthy seedlings.	Pre-emergence phase.	Post-emergence phases.	Total disease.	Post-emergence mortality (estimated).
First (6·6° C.)	Non-disinfected	90	79	9	11	20	5	94	80	4	14	18	7	96	73	4	23	27	16
	Disinfected	99	99	0	0	0	0	98	98	0	0	0	0	100	100	0	0	0	0
Second (8·2° C.)	Non-disinfected	97	84	3	13	16	7	95	82	4	13	17	5	100	73	0	27	27	13
	Disinfected	100	100	0	0	0	0	99	99	0	0	0	0	100	100	0	0	0	0
Third (9·7° C.)	Non-disinfected	99	90	0	9	9	3	100	87	0	13	13	3	98	82	1	16	17	3
	Disinfected	98	98	0	0	0	0	100	100	0	0	0	0	99	99	0	0	0	0
Fourth (11·9° C.)	Non-disinfected	100	94	0	6	6	1	100	88	0	12	12	3	98	83	1	15	16	4
	Disinfected	100	100	0	0	0	0	100	100	0	0	0	0	99	99	0	0	0	0
Fifth (13·6° C.)	Non-disinfected	98	90	2	8	10	2	100	93	0	7	7	0	89	71	10	18	28	7
	Disinfected	100	100	0	0	0	0	99	99	0	0	0	0	99	99	0	0	0	0

DISCUSSION

In this study of the epidemiology of *Helminthosporium* disease of oats, which was primarily planned for the purpose of evolving a standard technique for the evaluation of seed disinfectants, the findings of previous workers (MacKay, 1933; O'Brien and Prentice, 1930; O'Brien and Dennis, 1932; Turner, 1933) have been largely confirmed. It has been shown that under those conditions which provide a congenial environment for the growth of the young oat plant the primary phase of the disease is least severe, and that it becomes more intense as the environment is rendered less favourable. An interesting point which emerges is the fact that the disease tends to be more severe under conditions of relative drought (25 per cent. soil saturation) than when the soil is very wet (70 per cent. saturation), irrespective of temperature; an observation which has already been made by Leukel, Dickson, and Johnson (1933) for stripe disease of barley (*H. gramineum* Rabenh.). Such dry soil conditions are likely to occur but rarely in farm practice, especially in the wetter northern and western districts of Great Britain and Ireland where oats form the main cereal crop and where the soil moisture factor is not likely to be of primary importance. It is, however, common knowledge (O'Brien and Prentice, 1930; Turner and Millard, 1931) that the primary phase of the disease is of much greater importance in the colder and wetter parts of the country than in those which are warmer and drier, a fact which clearly indicates the main factor operating in farm practice to be that of soil temperature. That soil temperature is the main determining factor is further supported by the work of O'Brien and Dennis (1932) and by observations such as that of Davies in 1933, who suggests that the disease is of more importance in the highlands than in the lowlands of North Wales. It is also of interest to note that both Johnson (1925) and Smith (1929) consider low soil temperature to be of primary importance in the case of *H. gramineum* Rabenh. attacking barley. It can, therefore, be asserted that a high soil moisture content in itself is not conducive to the onset of the disease except in so far as the soil temperature is influenced by its moisture content. At the same time it should be borne in mind that under conditions of relative drought, which are not favourable for the rapid growth of the oat, a rise in soil temperature does not lead to the same progressive decrease in the incidence of the disease as occurs in moist and wet soils, soil moisture in such cases acting as a limiting factor.

The results obtained suggest that the onset of the disease in its worst form may be largely averted by deferring the sowing of the oat crop until the latest possible date, when in a normal season the soil temperature will have risen. This is true, but the position is further complicated in districts where frit fly (*Oscinella frit* Linn.) damage is likely, for this pest usually causes most injury in late-sown crops where the plants are in a susceptible stage of growth coincident with the appearance of the fly. The comparatively early sowing of spring oats is a recognized method for the control of frit fly injury (Smith, 1931), and it has been demonstrated by Chamberlain (1933) that early sowing under Northern Ireland conditions results in a much smaller infestation of the plants with the larvae than occurs in late sowings. When it is taken into account that Helminthosporium disease may be prevented by efficient seed disinfection prior to sowing, with the result that the seed may be sown as early as is convenient without risk of crop injury by the disease, then the way is made clear for the prevention of frit fly injury by such early sowing. The only conditions where seed disinfection was found to provide imperfect control of Helminthosporium disease were in dry soil at relatively high temperatures; circumstances which are not likely to occur with early sowings. Further, the sowing of disinfected seed in good time is likely to produce a crop of sturdy evenly developed plants which will, in a normal season, have outgrown the stage during which they are susceptible to frit fly attack by the time the fly appears.

It now remains to determine how the most suitable technique for the evaluation of seed disinfectants can be elaborated from the results obtained in this epidemiological study. In the first place it must be decided what phases of the disease shall be employed for disease assessment, and here every stress is laid upon the typical striping of the leaf, which is easily observed and less easily confused with symptoms resulting from other causes. In practice it has been found desirable to discard coleoptile stripe except when accompanied by leaf striping and to eliminate the pre-emergence phase as much as possible. The darkening of the vascular bundles of the coleoptile observed by Turner and Millard (1931) and the ease of confusing this with coleoptile stripe is the main reason for discounting this phase of the disease. The results obtained show that the pre-emergence phase occurs at a maximum when the mean soil temperature is low (6–8° C.) except in the case of dry soil, when it is also marked at high soil temperatures. They indicate further that a very high incidence of post-emergence phases easily identifiable as due to Helminthosporium attack, coupled with an almost negligible amount of the pre-emergence phase, occurs in plants grown under a mean soil temperature of from 8 to 10° C., which conditions would therefore appear to be most suitable for use in the evaluation of seed disinfectants. Under conditions obtaining in Northern Ireland a temperature mean approximating to this can be realized in most seasons by commencing the test during the second or third week in March, St. Patrick's Day (March 17) being a useful guide as to the date by which operations should be well under way. It is not necessary to use really wet soil for test purposes, as it has been shown that the disease occurs with equal severity in moist soil (approximately 50 per cent. saturation) which is easier to prepare and handle, while the soil type employed does not appear to be of primary importance. The test requires approximately 60 days. The fact that certain seed disinfectants have been found to be less efficient under conditions of dry soil and high temperatures makes it desirable that a further test be carried out later in the season. Here a mean soil temperature of from

12 to 13° C. is required with the soil moisture approximating 25 per cent. saturation. This test requires approximately 30–35 days, and the necessary temperature conditions can usually be obtained in Northern Ireland by commencing operations during the first fortnight in May. In addition to recording the behaviour of certain disinfectants under conditions of high soil temperature and low moisture the results from the second test serve as a useful check on those obtained by the first.

SUMMARY

1. An account is given of a study of the epidemiology and control of the primary phases of Helminthosporium disease of oats made by raising seedlings from infected seed (non-disinfected and disinfected) under varying conditions of soil type, temperature, and moisture.

2. Soil temperature has been shown to be the most important factor governing the extent to which the disease occurs when infected seed is sown. The temperature range used covers that likely to occur in a normal spring (early March to mid-May), and within the limits of this range a progressive decrease in the incidence of the disease occurs as the mean temperature rises and conditions for the rapid growth of the oat plant are improved. In moist or wet soil the moisture factor is not of primary importance, but under conditions of relative drought soil moisture behaves as a limiting factor, with the result that a temperature increase does not lead to the same corresponding decrease in the incidence of the disease. Soil type appears to affect the incidence of the disease only in so far as it may be influenced by the heating and moisture-retaining properties of the soil.

3. The pre-emergence phase of the disease is most marked under conditions of low soil temperature and also tends to occur under high temperature conditions in dry soil.

4. The disinfection of the seed prior to sowing with an organic mercury dust of proved worth secures almost perfect control of the disease under all conditions except when the soil is dry and the temperature high. In such cases the disinfectant used in this investigation proved to be slightly less effective.

5. For the oat variety Potato the results obtained hold for both heavily and lightly infected samples of seed grain.

6. From the results obtained a routine biological technique has been elaborated whereby, providing an infected sample of seed is used, a high incidence of the disease may be obtained with certainty. The adoption of this technique admits of the testing of seed disinfectants for the control of Helminthosporium disease under stringent conditions with greater accuracy and speed than has been found possible by the method of field trials.

ACKNOWLEDGEMENTS

The writer wishes to acknowledge the willing and valuable assistance rendered by Mr. T. N. Greeves, B.Sc., M.Agr., in connexion with the large amount of routine work involved in this investigation. He further thanks John Malone for his ready assistance and in particular for his help in the preparation of photographs.

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EXPLANATION OF PLATES XXIX AND XXX

Illustrating Mr. Muskett's paper on 'A Study of the Epidemiology and Control of Helminthosporium Disease of Oats'

PLATE XXIX

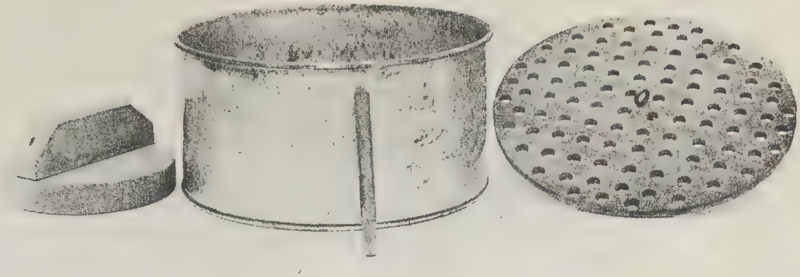
Fig. 1. Apparatus used in the preparation of each pot for sowing to ensure even distribution and equal depth of planting of the seed.

Fig. 2. A view of two pots. The seedlings here are almost ready for cutting and examination for the presence of the disease.

Fig. 3. A general view of part of the roof veranda where the work was carried out. A self-recording thermometer, the decimal scale used for weighing each pot, and the watering apparatus are shown in the photograph.

PLATE XXX

Photographs of the populations of diseased seedlings occurring in crops grown in the same soil from the same seed sample under varying conditions of soil temperature and moisture. This particular series illustrates the results obtained for sample Potato A grown in Stormont soil in 1934. The black line drawn below a number of the seedlings in each photograph indicates those seedlings which are likely to succumb to the attack. The two seedlings to the right in each photograph are healthy plants grown under similar conditions and included for purposes of comparison.



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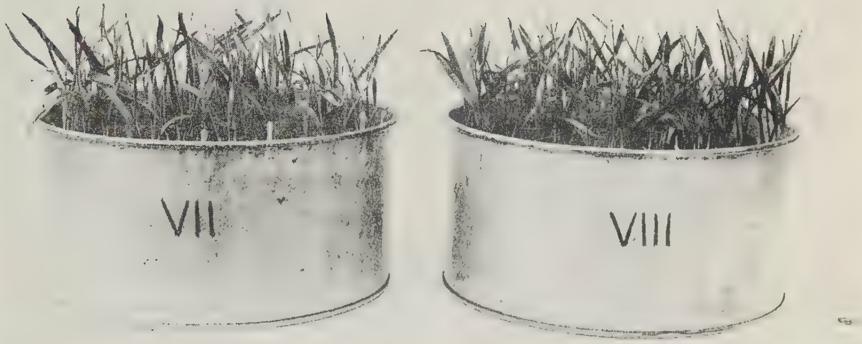

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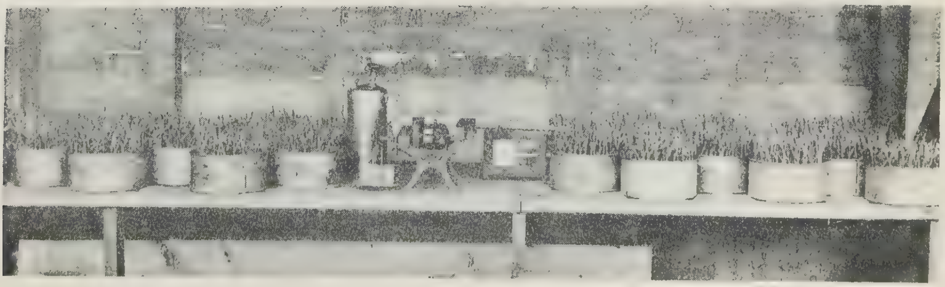
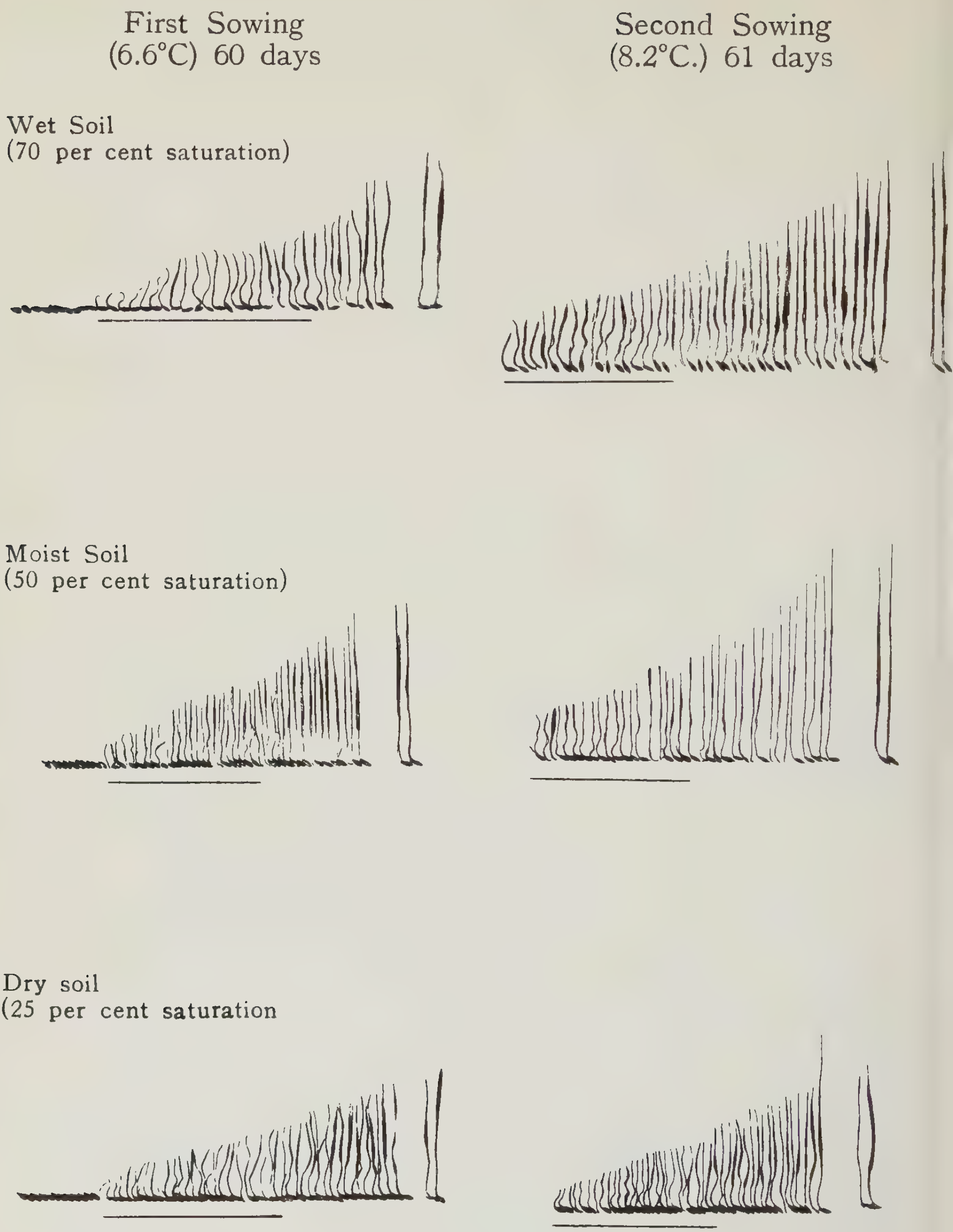


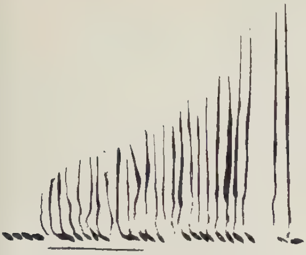
3

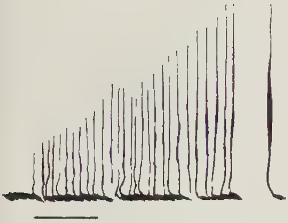
Huth Stubbs X. Kent

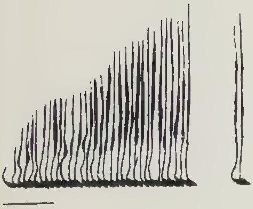
MUSKETT — HELMINTHOSPORIUM.


First Sowing
(6.6°C) 60 days
Second Sowing
(8.2°C.) 61 days
Wet Soil
(70 per cent saturation)
Moist Soil
(50 per cent saturation)
Dry soil
(25 per cent saturation

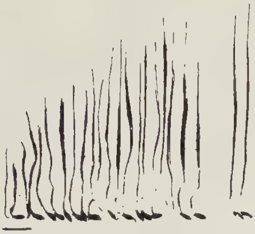
Third Sowing
(9.7°C.) 47 days

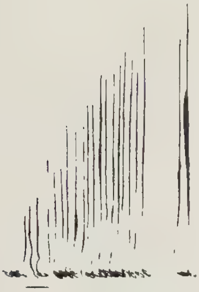


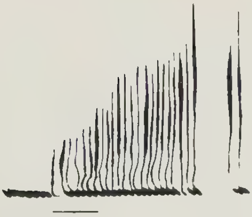




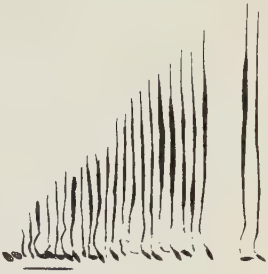
Fourth Sowing
(11.9°C.) 36 days

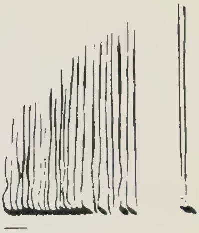


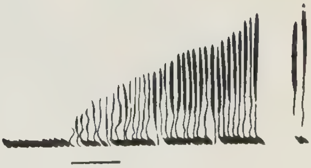




Fifth Sowing
(13.6°C.) 30 days







Huth, Stubbs X. Kent.

Cytological Studies of Pisum

I. Structural Hybridity in P. humile

BY

EILEEN SUTTON

(*John Innes Horticultural Institution, Merton*)

With Plate XXXI and twenty-five Figures in the Text

INTRODUCTION

THE object of this paper is to give an account of chromosome abnormalities found in *Pisum humile* and to present a quantitative analysis of chiasma formation so far as this is made possible by the occurrence of a particular kind of abnormality, namely inversion.

MATERIAL AND METHODS

The material used for this study came from two sister plants, $87^7/35$ and $87^8/35$, from among the progeny of one part-sterile plant, $1^3/34$, which was detected by Miss Pellew in 1934. It was the only sterile plant in a family of twenty-four, and no sterility had been noted in the few plants grown for three previous generations. Flower buds were fixed in Carnoy and La Cour's 2 B, the sections cut at 22–24μ and stained by the gentian-violet-iodine method. The drawings were made with a camera lucida, Text-figs. 6, 8, and 9 at an initial magnification of 3,500 diameters and the rest at a magnification of 3,000 diameters. All of them have been reduced to two-thirds of the original size in the text.

As the peculiarities of the two plants were quite different, the observations and conclusions with regard to each will be considered separately.

$87^7/35$. Inversions.

At metaphase of the first meiotic division most cells are normal with seven bivalents, but 8 per cent. of the total have only six bivalents with two univalents, or five bivalents with four univalents (Text-fig. 1). Evidence that chromosomes occasionally fail to pair and therefore fail to separate regularly at anaphase is also provided by the observation at later stages of lagging univalents (Text-fig. 7) and of daughter cells with unequal numbers of chromosomes (Text-fig. 8).

At first anaphase a chromatid bridge with a fragment is visible in about 60 per cent. of the cells. At late anaphase the bridge is attenuated (Text-fig. 2). Apparently it does not break, but it loses its staining properties; it is

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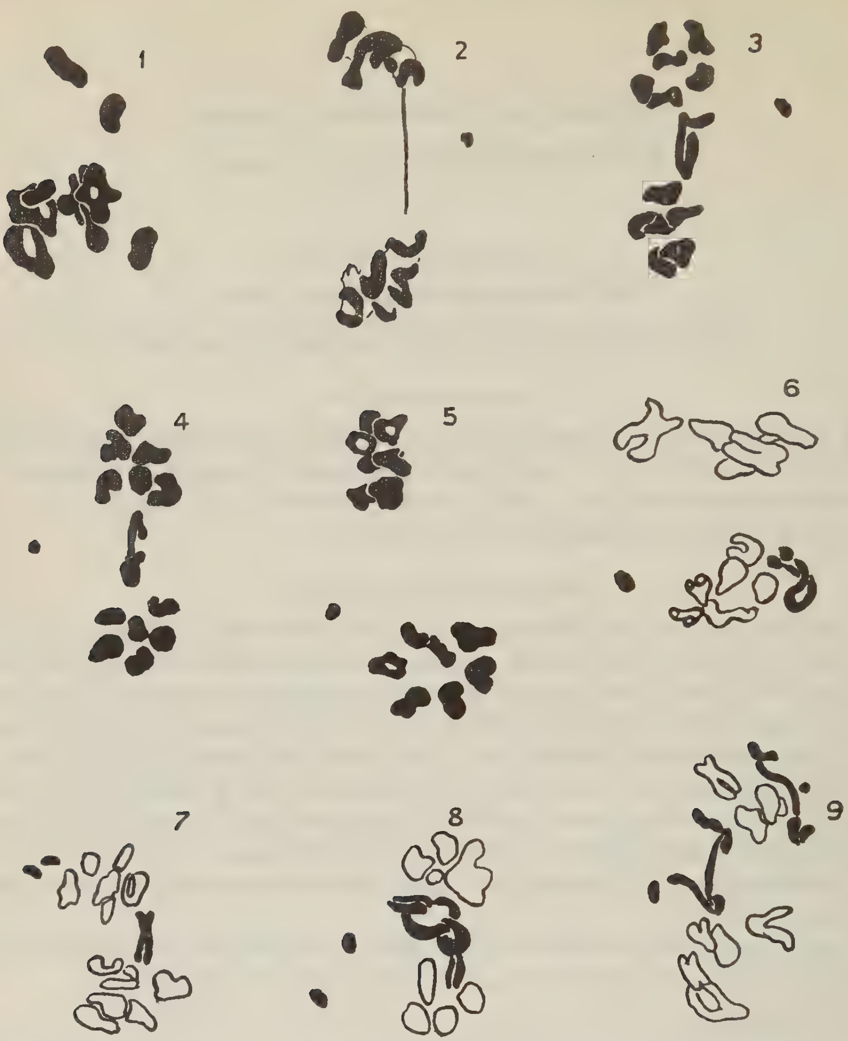
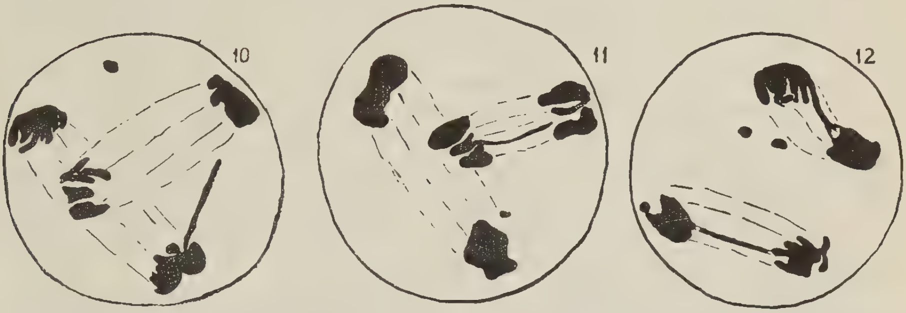


Text-figs. 1–9. Meiosis in $87^7/35$. (Figs. 6, 8, 9 ×2300, the rest ×2000). Fig. 1. First metaphase: 5 bivalents and 4 univalents. Fig. 2. First anaphase: chromatid bridge and small acentric fragment. Fig. 3. Second metaphase: bridge and large fragment. Fig. 4. Second metaphase: bridge and small fragment. Fig. 5. Second metaphase: large fragment, no bridge. Fig. 6. Second metaphase: large fragment and dicentric loop chromatid. Fig. 7. Second metaphase: two large fragments, no bridge, one lagging univalent. Fig. 8. Second metaphase: two large fragments and two interlocked loop chromatids. Fig. 9. Second metaphase: two bridges, with large and small fragments.

sometimes very faint at anaphase and telophase, nevertheless it is quite clear at second metaphase when the chromosomes of the daughter nuclei are re-orientated for the second division (Text-figs. 3 and 4 and Plate XXXI). At this stage most cells have one bridge and one fragment, which may be large

(Text-fig. 3) or small (Text-fig. 4); some have a fragment but no bridge (Text-figs. 5, 6); and in some of these a loop chromatid can be seen (Plate XXXI); two show two large fragments, the first cell having no bridge (Text-fig. 7 and Plate XXXI), the second having a configuration which will be described later (Text-fig. 8); and in one cell two separate bridges can be seen, each with an accompanying fragment, one large, one small (Text-fig. 9).

At second anaphase one can still see bridges formed at the first division. No cell-walls are formed during meiosis, so that the four daughter nuclei are included within one wall, and persistent first-division bridges pass obliquely between two of these nuclei (Text-fig. 10). There are, however, about 80 per cent. of the tetrads with bridges between two of their nuclei, while bridges were formed at only about 60 per cent. of the first divisions, so that about 20 per cent. of the tetrads must have bridges formed at the second division. These bridges pass directly between two sister nuclei (Text-fig. 11) and in one tetrad two such bridges have been observed, accompanied by two of the larger fragments (Text-fig. 12).



Text-figs. 10–12. Second anaphase in $87^7/35$. (×2000). Fig. 10. Persistent first-division bridge and large fragment. Fig. 11. Second-division bridge and small fragment. Fig. 12. Two second-division bridges and two large fragments.

Although it is not possible here to observe inverted pairing of chromosome segments at pachytene, the presence of dicentric bridges with acentric fragments at anaphase may be taken as evidence that such pairing has taken place, followed by chiasma formation in the inverted segments (Darlington, 1937). As the fragment formed by crossing-over in a given inversion is always the same size (the length of the inversion plus the sum of the lengths of chromosome distal to it) the occurrence of fragments of two different sizes shows that the plant was heterozygous for two different inversions.

The first- and second-division bridges and single or double fragments described above may be accounted for in the following ways:

1. A cell without a bridge or fragment at first anaphase has had no inversion chiasmata, or two reciprocal inversion chiasmata which cancel one another out.

2. A single dicentric bridge with a fragment at the first division will result from a single inversion chiasma, or from two disparate inversion chiasmata,

so long as there are no chiasmata, or only comparate chiasmata, in the proximal region (i.e. between the inversion and the centromere). See Text-figs. 2, 3, 4, 9, 10; Plate XXXI.

3. A loop chromatid (not a bridge) and a fragment at the first division will result from a chiasma or chiasmata of type 2 in the inversion, with a disparate proximal chiasma. The loop will form a bridge between a pair of sister nuclei at second anaphase. See Text-figs. 5, 6, 11; Plate XXXI.

4. Two loop chromatids and two equal fragments at the first division, and consequently two second-division bridges, one between each pair of sister nuclei, will result from two complementary chiasmata in the inversion with a third, disparate, chiasma proximal to the inversion. See Text-figs. 7, 8, 12; Plate XXXI. The two loops are not visible in Text-fig. 7, but their existence is inferred from the two equal fragments; similarly in Text-fig. 12 their formation is inferred from the equal fragments and the two bridges which have resulted at second anaphase. In Text-fig. 8, however, the complicated configuration between the two groups of chromosomes is believed to represent two interlocking loops. Upcott (1937, Fig. 51) has found such interlocking, and shows how it might occur as a result of complementary chiasmata in an inverted region with a disparate proximal chiasma.

An attempt has been made to estimate the number and relationships of chiasmata formed in and proximal to each inversion, and the proportion of inversion crossing-over to crossing-over in the chromosome complement as a whole. This estimate is necessarily rough, because in sectioned material cut cells are liable to misinterpretation—for example, a fragment which has been thrown out to the side may easily be cut away, and if there is no bridge the cell will then be interpreted as of type 1 listed above, whereas it would really be of type 3.

The counts have been made from second metaphase cells, these being the most numerous.

Table I gives the numbers obtained for the inversion giving the larger fragment.

TABLE I

Cells with no bridge and no fragment	66	44 per cent.
Cells with one bridge and one fragment	65	43·3 ,,
Cells with one fragment and no bridge	17	11·3 ,,
Cells with two fragments and no bridge	2	1·3 ,,
Total	150	

If disparate and comparate double chiasmata occur in the inversion with equal frequency, there should be as many reciprocal double chiasmata (giving no bridge or fragment) and twice as many disparate double chiasmata (giving one fragment, with or without a bridge) as there are complementary double chiasmata, which are distinguishable by the two fragments formed. The number of cells with two fragments should therefore be multiplied by four to obtain the number of double cross-overs in the inversion, although with such small numbers the probable error of the result will be large. Table II gives the number of double inversion chiasmata calculated in this way and the number of cells with one inversion chiasma, obtained by deducting the expected number with disparate double chiasmata from the total number of cells with one fragment (with or without a bridge). It is assumed that triple chiasmata do not occur, since even double chiasmata are very rare.

TABLE II

(*a*)	Cells with one chiasma in inversion	78	
	Cells with two chiasmata in inversion	8	
	Total number of chiasmata in inversion (150 cells)	94	
	Average number of chiasmata in inverted region, per cell	0·60	
(*b*)	Cells with chiasma in inversion: proximal chiasmata none or comparate	65	(77 per cent.)
	Cells with chiasma in inversion: proximal chiasma disparate	19	(23 ,,)

Again, if we assume that disparate and comparate chiasmata occur in equal numbers and only one proximal chiasma per cell is formed, it follows that proximal chiasmata are formed in 46 per cent. of the cells with an inversion chiasma. Incidentally the percentage of all cells (with or without an inversion chiasma) which have chiasmata in the proximal region may be taken to be the same, or slightly higher to allow for a reduction due to the interference when there is also an inversion chiasma. There may be more than one chiasma in the proximal region, however, so that it is not possible to compare the lengths of the inverted and proximal regions by the number of chiasmata formed in them.

In Table III the data relating to the inversion with the smaller fragment are given.

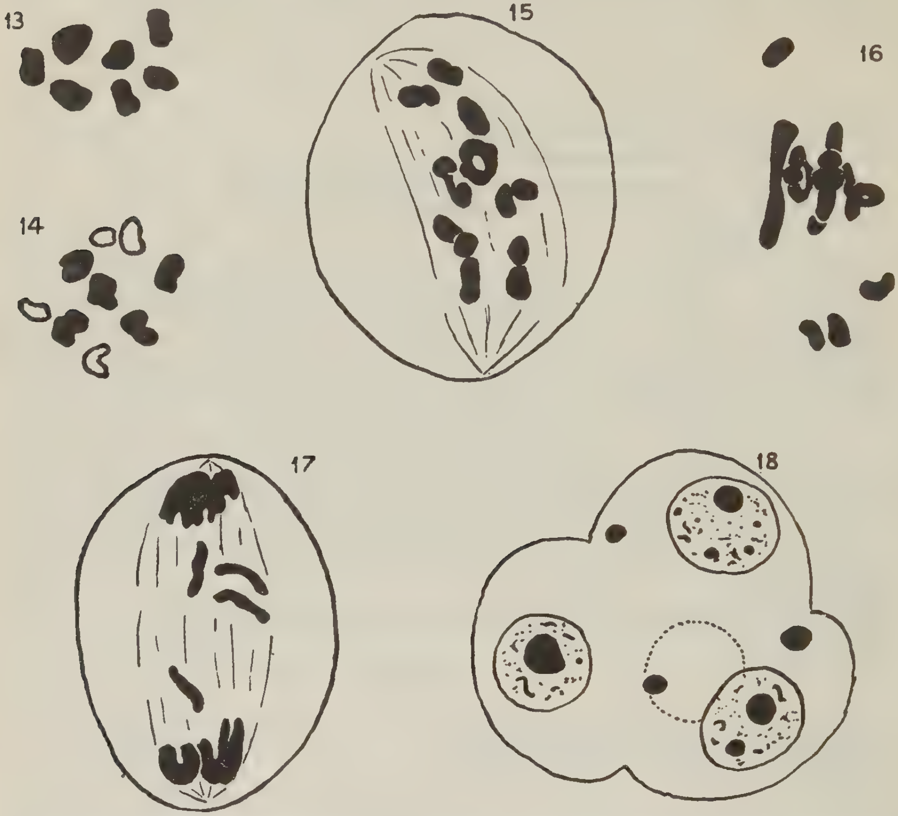
TABLE III

Cells with no bridge and no fragment	119	79·3 per cent.
Cells with one bridge and one fragment	23	15·3 ,,
Cells with one fragment and no bridge	8	5·3 ,,
Total	150	

As no signs of complementary double crossing-over were observed, and crossing-over in this inversion is less frequent than in the other, it is unnecessary to make corrections. The average number of chiasmata per cell in this inversion is 31/150 or 0·21, the proportion of disparate proximal chiasmata is 8/31 or 26 per cent., and the expected proportion of all proximal chiasmata is therefore 52 per cent. The figures therefore indicate that this inversion may be rather farther from the centromere than the other, and that it is considerably shorter.

In some species of Tulipa heterozygous for inversions there is an excess of second-division bridges, and it appears that disparate and comparate chiasmata are not formed at random, but that the former are more frequent (Upcott, 1937). There is no evidence that chiasma relationships in *P. humile* are not random.

In 150 cells the total number of chiasmata in the inversions is 125, and the frequency of inversion crossing-over per cell is therefore 0·83. As the average chiasma frequency per cell (calculated from 10 cells) is 19·7, the coefficient of hybridity due to inversion in this plant is 0·83/19·70 = 0·04.

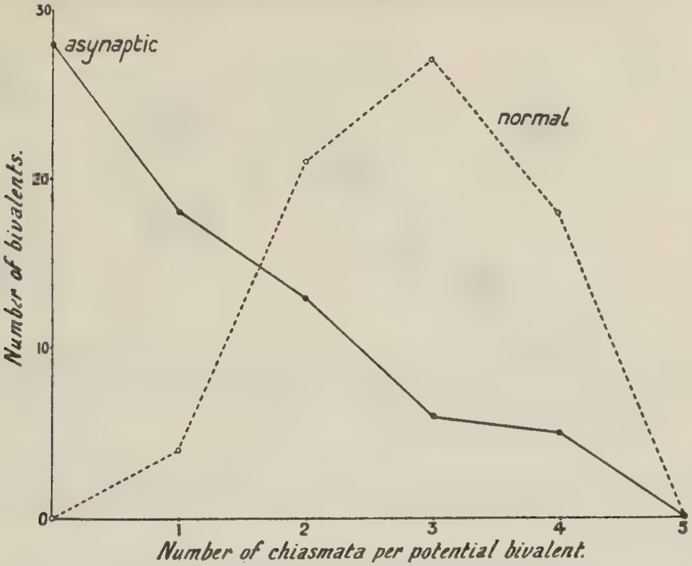


TEXT-FIGS. 13–18. Meiosis in $87^8/35$. (×2000). Fig. 13. First metaphase, polar view: seven bivalents. Fig. 14. First metaphase, polar view: five bivalents and four univalents (in outline). Fig. 15. First metaphase, side view: two bivalents and ten univalents, curved spindle. Fig. 16. First metaphase, side view: five bivalents and four univalents. Fig. 17. First anaphase: division of lagging univalents. Fig. 18. Second telophase: tetrad with micronuclei.

$87^8/35$. 'Asynapsis' and other abnormalities.

The first peculiarity found in this plant was that there are a variable number of univalents in the first metaphase cells (Text-figs. 13–16 and Plate XXXI). A few cells with the full number of seven bivalents have been seen (Text-fig. 13) and one with no bivalents. Between these extremes the number of cells with a given number of bivalents is fairly evenly distributed about a mode of five (Table IV).

The chiasma frequency is reduced to 1·2 per potential bivalent as compared with 2·8 in a normal plant (average of 10 cells) and the proportion of bivalents with more than two chiasmata is very low (Table V and Fig. 19).



TEXT-FIG. 19. Graph showing comparison of chiasma frequency in normal (broken line) and asynaptic (unbroken line) plants of *P. humile*.

TABLE IV

Count of 100 Cells at First Metaphase

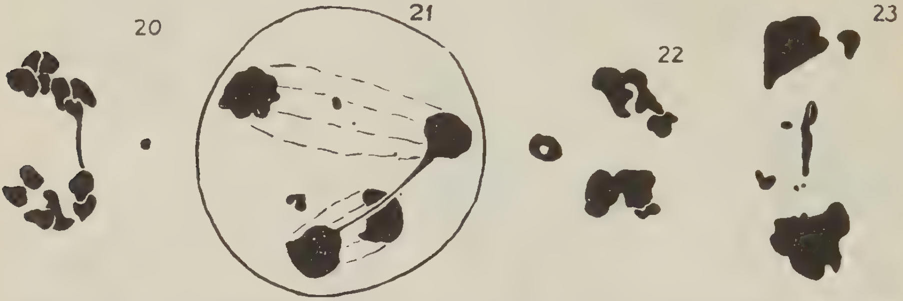
No. of bivalents . .	0	1	2	3	4	5	6	7
No. of cells . . .	1	2	8	10	17	38	17	7

TABLE V

Comparison of Number of Bivalents with a Given Number of Chiasmata in $87^8/35$ and in a Normal Plant ($820^1/35$)

						Total.	
Chiasmata per bivalent . .	0	1	2	3	4	Cells.	Bivalents.
No. of bivalents, $87^8/35$ . .	28	18	13	6	5	10	70
„ „ 820^1 . . .	—	4	21	27	18	10	70

The spindle is elongated and slightly curved in some of the cells with a large number of univalents (Text-fig. 15). At first anaphase, univalents lag on the plate and occasionally divide (Text-fig. 17 and Plate XXXI). They are often excluded from the daughter nuclei, and besides the four nuclei of the pollen tetrad there are often micronuclei formed from these excluded univalents (Text-fig. 18). These characteristics are like those of 'asynapsis' in maize (Beadle, 1930, 1933) and *Datura* (Bergner, Cartledge, and Blakeslee, 1934), a condition which was found in each case to be determined by a single recessive mutation. Beadle found that the univalents in maize resulted from suppression of chiasma formation, and this in turn was due to incomplete pairing at pachytene. Probably 'asynapsis' in *P. humile* is also due to a gene mutation causing reduction of chiasma formation; evidence of this is that when bivalents are formed they have a low average chiasma frequency. There is no proof, however, that this occurs on the female side also, as most of the buds were fixed and the plant set no pods there is no indication of the amount of sterility.



Text-figs. 20–3. Meiosis in $87^8/35$. (×2000.) Fig. 20. Second metaphase: bridge and acentric fragment. Fig. 21. Second anaphase: persistent first-division bridge, fragment, and univalent. Fig. 22. First telophase: chromatid ring. Fig. 23. First telophase: 'univalent bridge' with fragment and two univalents.

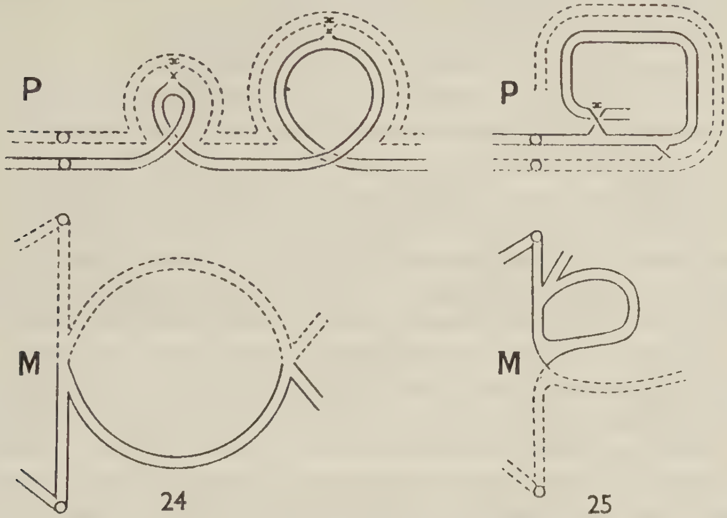
There is also evidence that this plant was heterozygous for different structural changes in the chromosomes:

1. Two bridges (each with a fragment) have been seen at second metaphase (Text-fig. 20) and one persistent first-division bridge was seen at second anaphase (Text-fig. 21). The plant was heterozygous for a small inversion in which perhaps 1 per cent. of crossing-over occurred.

2. At anaphase-telophase of the first division three cells have been found with chromatid rings excluded from the daughter nuclei (Text-fig. 22 and Plate XXXI). It is assumed that these rings are either acentric fragments or centric univalents. A continuous ring fragment could be formed from crossing-over in each of two inversions in the same arm of a chromosome, or from crossing-over between reduplicated segments in a single chromatid (Text-figs. 24, 25) both of which have been found by Upcott in Tulipa species (1937); a centric ring univalent could be formed only by a duplication which enabled the ends of a single chromosome to pair and form a chiasma (Richardson, 1936, Diagram 5). The double inversion would also give a bridge and a second fragment, however, so that the rings seen here are more probably due to duplication.

3. In one cell at first telophase there are two ordinary univalents lying outside the daughter nuclei and also a chromatid bridge extended between them with a fragment lying beside it (Text-fig. 23 and Plate XXXI). This resembles the univalent bridges found by Upcott (1937) in triploid Tulipa species and attributed to inversion crossing-over with a disparate proximal

chiasma in a trivalent, followed by lagging of the chromosome which has taken part in both chiasmata. If the bridge shown in Text-fig. 24 arose in the same way there must have been pairing between two non-homologous bivalents, or between a univalent and a non-homologous bivalent, and this implies a previous translocation of a segment from one chromosome to another non-homologous chromosome. On the other hand, Darlington (1936) found that a bivalent which lies off the metaphase plate will lag and divide late, and one of the daughter chromosomes may proceed to divide again before the second division of the other chromosomes. If this happened in a bivalent where inversion and disparate proximal crossing-over had already occurred, a univalent bridge would result. This is perhaps a more likely occurrence here than multiple pairing, since unorientated bivalents are sometimes seen at metaphase, whereas no multiple associations have been observed.



TEXT-FIGS. 24, 25. Diagrams showing pairing relationships which produce an acentric ring fragment. Fig. 24. Reciprocal chiasmata (*x*, *x*) in two different inversions in the same arm of a chromosome. Fig. 25. Chiasma (*x*) between two reduplicated segments of the same chromatid. P = pachytene. M = metaphase.

ORIGIN OF STRUCTURAL CHANGES IN PISUM

Most of the structural changes found in the chromosomes of Pisum species have been due to interchange of segments between non-homologous chromosomes, several such changes having been recorded in *P. sativum* (Håkansson, 1929, 1934; Sansome, 1929; Sutton, 1934). Håkansson (1934) has found what may be a simple translocation in *P. sativum*. He has also described the results of two *P. humile* × *P. arvense* crosses (Håkansson, 1936); one showed that, compared with the *P. arvense* parent used, the *P. humile* parent had two chromosomes with interchanged segments; the second cross showed that, besides an interchange, the *P. humile* parent had one chromosome with a segment inverted relative to that of the *P. arvense* parent. Crosses between *P. sativum* and an inbred line of *P. humile* grown at this Institution have revealed a complicated double translocation system from which chromosomes with duplicated end-segments have been derived (Sansome, unpublished).

Most of these chromosome changes have been detected by means of crosses between stocks which were structurally homozygous, but which gave structurally heterozygous and partially sterile hybrids. In some cases, however, partially sterile plants have arisen apparently spontaneously in fertile lines which have not been crossed, and these plants have proved to be structural hybrids; of these, the parent of the plants considered here is an example.

P. humile has been grown frequently in small numbers (6–12 plants, self-fertilized) since it was introduced to this country from Palestine by Arthur Sutton (*c.* 1908). No conspicuous gametic sterility was noted before 1934, although occasional ovules failed. A hybrid from a natural cross with *P. sativum*, which has also been grown here, would be easily distinguishable from pure *P. humile*, and a natural cross between any of the *P. humile* plants grown in one year would be a cross between plants of a highly inbred line. It must therefore be assumed that a number of genetic and structural changes have taken place spontaneously within this line. These changes may have occurred more or less simultaneously in the parent plant $1^3/34$. On the other hand, it is possible that the asynaptic mutation arose first, and the other changes followed from irregular pairing (intra-haploid pairing, Darlington, 1937) in an asynaptic plant where normal pairing was already upset. The asynaptic plant studied was scored as '? fertile' judged by its pollen, so that presumably unbalanced pollen grains degenerate at an early stage and the apparent sterility of the plant is not very great. The mutation might therefore have occurred, without being detected, a generation before any marked sterility was noticed.

SUMMARY

A description is given of chromosome behaviour at first metaphase and later stages in two sister plants of *Pisum humile*.

One of these plants was heterozygous for two inversions, and an analysis is made of chiasma formation in and proximal to the inverted segments. The formation of comparate and disparate pairs of chiasmata appears to be random.

The second plant was 'asynaptic' owing to reduced chiasma formation, and was also heterozygous for various structural changes. The abnormalities described arose spontaneously in a single inbred line.

ACKNOWLEDGEMENTS

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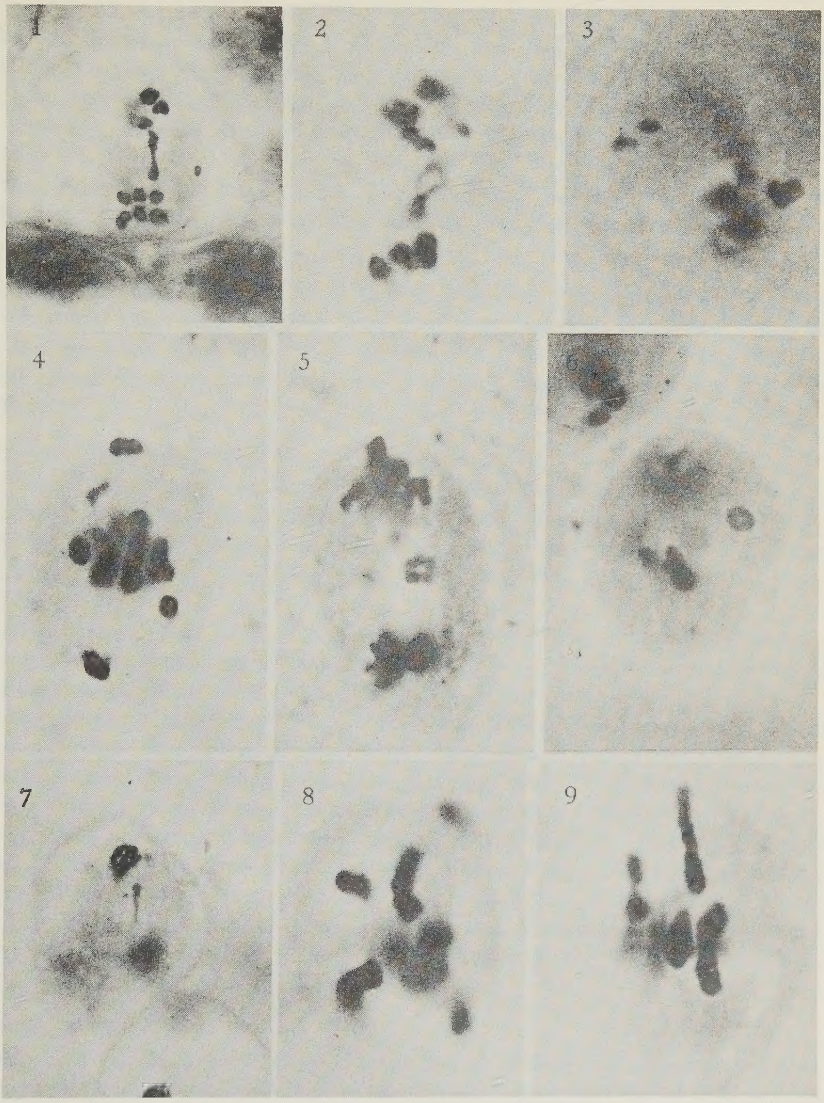
EXPLANATION OF PLATE XXXI

Illustrating Miss Eileen Sutton's paper 'Cytological Studies of Pisum. I. Structural Hybridity in *P. humile*'.

Figs. 1–3. $87^{7}/35$. Fig. 1. Second metaphase: bridge and fragment. Fig. 2. Second metaphase: dicentric loop chromatid. Fig. 3. Second metaphase: two equal fragments. Figs. 4–9. $87^{8}/35$. Fig. 4. First metaphase: five bivalents and four univalents. Fig. 5. First anaphase, showing a dividing univalent. Fig. 6. First telophase, showing ring fragment or univalent. Fig. 7. First telophase: fragment and 'univalent bridge'. Figs. 8, 9. First metaphase: bivalents lying off the plate. Figs. 1 and 7, magnification ×1000; the rest ×1700.



Huth, Stubbs X., Kent.

SUTTON—CYTOLOGY OF PISUM.

NOTE

THE RESISTANCE TO POISONS OF DESICCATED PLANT TISSUES.—Some time ago (this Journal, Oct. 1930) an account was given of work on the moss *Mnium Hornum*, which showed that, while in a thoroughly dried condition, treatment for a limited time with certain toxic substances was not fatal.

Further types have since been used, and brief details of those which were found to withstand treatment with the toxic substances indicated, are now given:

Moss—Hylocomium squarrosum.

Alcohol—Material survived after 14 hours' immersion in absolute ethyl alcohol. *Xylol*—Material subjected first to 1 hour in absolute alcohol, survived 4 hours in xylol. *Acetone*—Result as with xylol. *Ether*—Result as with xylol. *Chloroform*—Material previously subjected to 1 hour in absolute alcohol survived 2 hours in chloroform.

Lichen—Lecanora conizaeoides.

The lichen was treated to obtain it in a thoroughly pulverulent condition. Preliminary experiments indicated that a safe method of deciding whether the material had survived treatment, was to examine it after three weeks culture, following the treatment. A bright green colour in the algal elements was taken as indicating survival.

Alcohol—Material was subjected to a range of exposures to alcohol. About 25 per cent. of the algal cells survived 4 hours in alcohol, 10 per cent. or less survived 8 hours in alcohol, and only about 0·1 per cent. survived 21 hours.

Acetone—In some cases, material was placed in alcohol for a short time before immersion in acetone, while in others no such prior treatment in alcohol was given. 8 hours in acetone gave about 15 per cent. survival, and the percentage fell to 10 per cent. for 12 hours, 5 per cent. for 18 hours, and about 0·1 per cent. for 24 hours. *Ether*—A short preliminary treatment with alcohol was given. Not more than 2 per cent. of the cells survived after 4 hours in ether. *Xylol*—Material was treated with alcohol for 15 minutes prior to immersion in xylol. Survival ranged from 25 per cent. after 4 hours xylol, through 20 per cent. for 8 hours, 18 per cent. for 12 hours, about 8 per cent. for 18 hours, to a maximum of 30 hours from which treatment practically no cells recovered.

Bacillus coli communis.

Alfred Beyer (Zeitschrift für Hygiene 1912) had noticed that 'Absolute alcohol has a preservative effect on bacteria if all moisture is kept away.' He says of alcohol that 'Higher concentrations (than 70 per cent.) are ineffective (for purposes of disinfection) on account of a lack of water, as it leads to a more or less strong drying up and preserving of the bacteria.' Beyer worked chiefly on *Staphylococcus aureus*.

The importance of his paper lies in the fact that he showed bacteria to be capable

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of withstanding concentrations of alcohol other than absolute alcohol. While it is impossible to state with certainty that absolute alcohol actually penetrates the cell, no one probably would doubt that (say) 80 per cent. is capable of penetration.

Work was carried out with *Bacillus coli communis*, using concentrations of 90 per cent., 95 per cent., and so-called absolute alcohol, and in all cases the time allowed was half an hour.

Briefly, it may be stated that in all cases, a positive result was obtained, although the lower concentrations of alcohol were slightly more toxic. An immersion in absolute alcohol of 2 hours appeared to have no adverse effect on the bacteria. This, however, is not so important as is the fact that bacteria can survive treatment with dilute alcohol, since the probability is strongly in favour of the penetration of the latter.

Saccharomyces cereviseae (*Baker's yeast*).

This yeast is not usually exposed to periods of drought, but it was felt that, if it withstood desiccation and treatment with toxic substances, the presumption would be that other yeasts would survive similar treatment.

The yeast was obtained in a very finely divided condition by mixing it thoroughly with about 30 times its bulk of very fine sand, until practically all the cells were separate. After treatment the cells were cultured on nutrient gelatine, and evidence of survival was obtained by the budding of the cells within a few hours.

Alcohol—Material survived 192 hours in absolute alcohol, practically every cell producing a morula. *Ether*—Alcohol was used first and the yeast then withstood 42 hours in ether.

Acetone—After immersion in alcohol, the yeast survived 312 hours in acetone. Material budded equally well, whether treated previously with alcohol or not.

Chloroform—Material budded readily after 15 hours in chloroform.

In all the above cases, it is not suggested that the time indicated is the maximum which the yeast can withstand; and in all cases, practically every cell budded readily.

It is impossible to say whether the substances used penetrated the cells. No definite claim is made either way, though the results obtained from *B. coli* may be taken as in favour of penetration.

W. W. ALLEN.

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